cDNA CLONING AND TRANSCRIPTIONAL REGULATION OF THE VITELLOGENIN RECEPTOR FROM THE IMPORTED FIRE ANT, *SOLENOPSIS INVICTA* BUREN (HYMENOPTERA: FORMICIDAE)

A Dissertation

by

MEI-ER CHEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Entomology

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ABSTRACT

cDNA cloning and Transcriptional Regulation of the Vitellogenin Receptor from the Imported Fire Ant, *Solenopsis invicta* (Hymenoptera: Formicidae).

(December 2003)

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Co-Chairs of Advisory Committee: Dr. Larry L. Keeley
Dr. Patricia V. Pietrantonio

Receptors that transport vitellogenin into oocytes are of vital importance to egg-laying species because they promote oocyte development. In this study, we describe the cloning of the first hymenopteran vitellogenin receptor (VgR) cDNA. Using reverse transcription polymerase chain reaction (RT-PCR) and both 5'- and 3'- rapid amplification of cDNA ends (RACE), cDNA fragments encompassing the entire coding region of a putative VgR from fire ant (= *Si*VgR) were cloned and sequenced. The complete *Si*VgR cDNA has a length of 5764 bp encoding a 1782-residue protein with a predicted molecular mass of 201.3 kDa. The deduced amino acid sequence of the *Si*VgR revealed that it encoded a protein belonging to the low-density lipoprotein receptor superfamily. The number and arrangement of modular domains of *Si*VgR are the same as those of mosquito and fruit fly VgRs, except there are only four Class A cysteine-rich repeats in the first ligand binding domain of *Si*VgR compared to five in the mosquito and fruit fly. The deduced amino acid sequence of the *Si*VgR exhibited 35% and 31% identity to those of the mosquito and fruit fly VgRs, respectively. Northern blot analysis demonstrated that the 7.4-kb *Si*VgR mRNA was present only in

Northern blot analysis demonstrated that the 7.4-kb *Si*VgR mRNA was present only in ovaries of reproductive females – both alates (virgins) and queens (mated) and was more abundant in alates. The developmental profile of transcriptional expression was determined by semiquantitative RT-PCR. It showed that the *Si*VgR transcript increased 6-fold from 0- to 10-days after mating, then remained constant through 30 days. It also showed that the *Si*VgR transcripts increased with age in alate virgin females. The transcriptional expression of the *Si*VgR was up-regulated more than two-fold by methoprene, a juvenile hormone analog, as determined by using an *in vitro* system. This suggested the *Si*VgR gene is JH regulated.

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To Danielle Lewis and Yolande Yang, I offer my gratitude and friendship. They saw me at my worst and my best and accepted me through it all. I also thank all of my friends and lab mates who gave me all kinds of assistance. Thanks also to my family for everything they have done for me along the way.

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CHAPTER I

INTRODUCTION

Since its introduction into the southeastern USA, the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), has established itself as a serious urban, agricultural and medical pest of the south, and it is now expanding its range into additional northern and western states (Vinson, 1997). The total annual costs of damages and expenditures attributed to the fire ants in Texas only are about 1200 million dollars. (Texas A&M University, 2001).

Mating flights are the primary means of colony propagation. Mating flights occur in the proper environmental conditions. Usually, flights begin around 10:00 AM, one or two days after a rain, and when the temperature is above 24°C, it is not too windy, and it is generally sunny. Just before the mating flight, workers become very active on and around the surface of the nest and open small holes on the mound surface into the nest's interior. Alate males first emerge from these openings and fly away or first climb surrounding vegetation to facilitate flight. Alate females begin to emerge an hour or so later and join the males in flight (Vinson, 1997). Mating occurs during flight and the males die soon after mating with females. Once the alate females have mated, they land, dealate and lay eggs within 24 hours.

This dissertation is presented in the style of the Insect Biochemistry and Molecular Biology.

Colonies of imported fire ants are divided into two types based on the number of queens. Monogyne colonies contain a single queen, while polygyne colonies contain multiple, inseminated functional queens. In studies characterizing egg production, Fletcher *et al*. (1980) reported that physogastric (enlarged abdomen) queens in monogyne colonies produced about 200 eggs per day. In contrast, an individual polygyne queen may produce only 20 or 30 eggs per day. Queens from polygyne colonies are less physogastric and lay fewer eggs per individual than queens from monogyne colonies, but total egg production in polygyne colonies is several-fold higher than in monogyne colonies due to the presence of multiple reproductive queens (Fletcher et al., 1980; Greenberg et al., 1985; Vargo and Fletcher 1989).

The *S. invicta* queen is the center of colony existence. She controls the colony through the production of eggs by controlling the type of eggs she produces and by releasing pheromones that influence the physiology and behavior of the workers and other reproductives (Vinson, 1997; Vargo, 1998). There are three types of eggs. Sterile or trophic eggs are eaten, fertilized eggs produce sterile female workers or females capable of reproduction and unfertilized eggs develop into males (Vinson, 1997). In colonies, workers are completely sterile, but alate virgin females are capable of dealating and laying unfertilized (haploid) eggs. In normal colonies, a queen primer pheromone prevents alate virgin females from shedding their wings and developing their ovaries. However when removed from queen pheromonal influences, alate virgin females shed their wings and ovaries develop (Fletcher and Blum, 1981, 1983; Vargo, 1999). Topical applications of synthetic juvenile hormone (JH) I, II and III (Kearney et al., 1977) or the JH mimic, methoprene (Vargo and Laurel, 1994), induce dealation and

ovary development, even in the presence of the queen (primer pheromone present). This suggests that the queen pheromone may act by suppressing JH titers in co-habiting alate virgin females (Vargo and Laurel, 1994).

Vitellogenin (Vg) is the general name for a unique group of proteins that are synthesized extra-ovarially, transported to the ovaries, taken up and become the major egg yolk protein, vitellin (Vn) (Hagedorn and Kunkel, 1979). In most insects, Vgs are large oligomeric glycolipophosphoproteins consisting of two or more subunits. They are synthesized in the fat body as single or multiple precursors before being processed for secretion into the hemolymph. After Vgs are selectively taken up by the oocytes, they are stored in a crystalline form and referred to as Vns. Receptor-mediated endocytosis of Vg deposition into the oocyte has been studied in the blood sucking bug, *Rhodnius prolixus* (Oliveira et al., 1986), the cockroach, *Nauphoeta cinerea* (Kindle et al., 1988), the German cockroach, *Blattella germanica* (Konig et al., 1988), the locust, *Locusta migratoria* (Ferenz and Lubzens, 1981), the saturniid moth, *Hyalophora cecropia* (Kulakosky and Telfer, 1987), the tobacco hornworm, *Manduca sexta* (Osir and Law, 1986), the mosquito, *Aedes aegypti* (Koller et al., 1989), and the fruit fly, *Drosophila melanogaster* (Schonbaum et al., 1995). It is likely the mode of action for Vg uptake in *S. invicta*, as well.

The pathway of Vg internalization and Vg receptor (VgR) recycling has been studied (Fig. 1-1). Coated vesicles, the cellular structures associated with selective endocytosis were first observed in *A. aegypti* (Roth and Porter, 1964). In 1997, Snigirevskaya *et al.* reported the route of Vg and VgR during Vg internalization in *A. aegypti* oocytes. At the onset of Vg accumulation into the oocyte, the follicle cells that

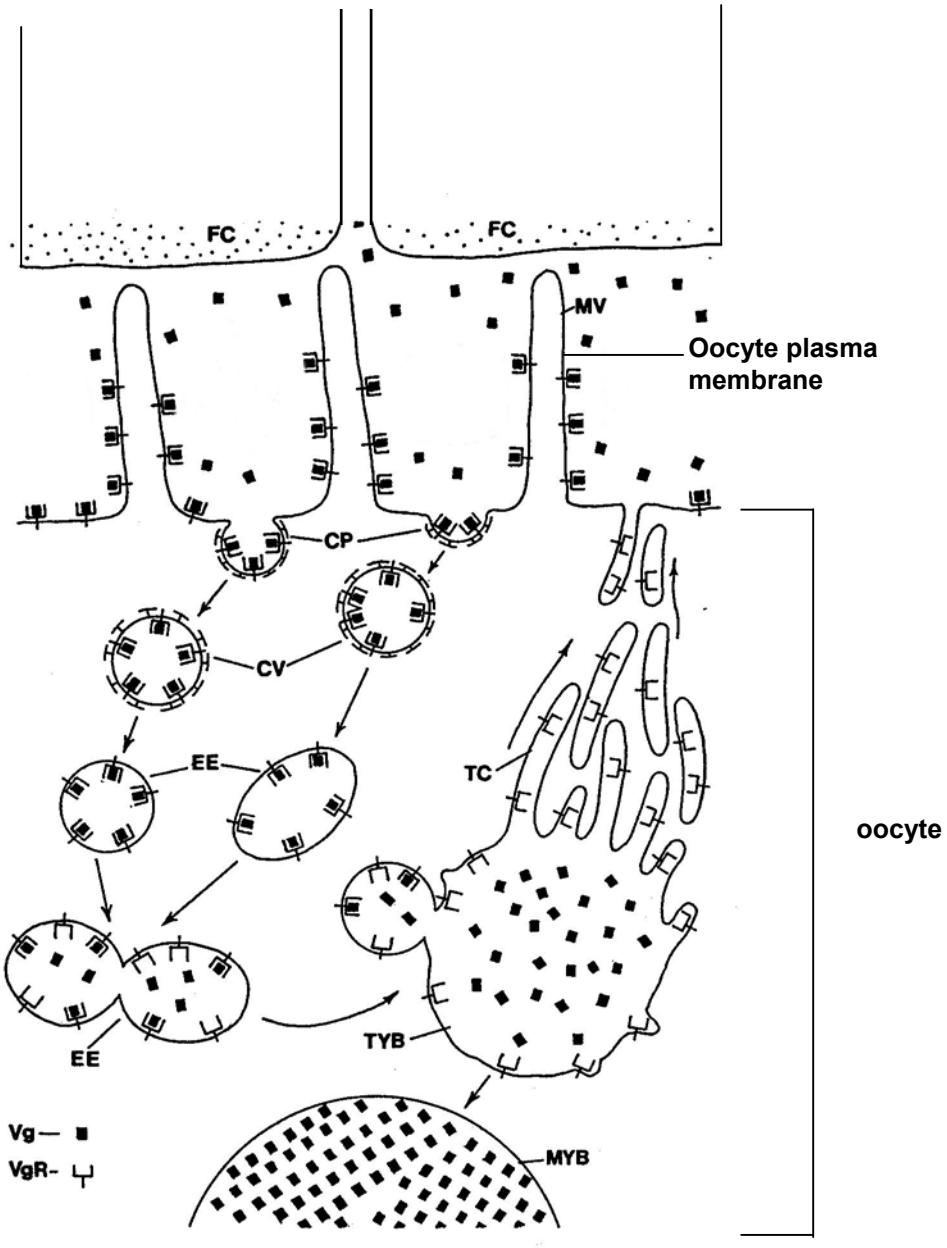

Fig. 1-1. Schematic interpretation of the endocytotic pathway and subsequent route of Vg and VgR during Vg internalization by mosquito oocytes. FC: follicle cell; MV: microvillus; CP: clathrin-coated pit; CV: coated vesicle; EE: early endosome; TYB: transitional yolk body; TC: tubular compartments; MYB: mature yolk body (modified after Snigirevskaya et al., 1997).

surround the oocyte, shrink and develop extensive intercellular spaces. Vg is delivered to the oocyte in the hemolymph through these intercellular spaces and binds to its receptor which is located in the binding microdomains of the oocyte plasma membrane. Vg/VgR complexes cluster in the clathrin-coated pits, which invaginate into the cytoplasm and pinch off to form coated vesicles. After losing their clathrin coats, the vesicles are transformed into early endosomes which fuse with endosomes to form late endosomes or transitional yolk bodies (TYB). Dissociation of Vg from its receptor likely occurs simultaneously with fusing of the early endosomes and their transformation into TYBs. In the TYB, the sorting and accumulating compartment, Vg is found free inside the vesicular reservoir, whereas VgR remains in the TYB membrane. The recycling of VgR from TYB to the oocyte surface occurs via the tubular compartment. In contrast, Vg is delivered to the mature yolk bodies where it is crystallized and stored until the onset of embryonic development.

Molecular cloning of VgR genes is necessary for further analyses of the mechanisms underlying Vg accumulation in insect oocytes. At the beginning of this work only two dipteran VgRs cDNA have been sequenced, the mosquito and the fruit fly, *A. aegypti* and *D. melanogaster*, respectively (Schonbaum et. al., 1995; Sappington et al., 1996). Both the VgRs of *A. aegypti* and *D. melanogaster* are members of the low-density lipoprotein receptor (LDLR) superfamily, and they share 42% amino acid identity and 63% similarity to each other (Sappington et al., 1996). By the end of this work, there are two more known insect VgR sequences, the American cockroach, *Periplaneta americana* (accession number BAC 02725) and the mosquito *Anopheles gambiae* (accession number XP_310672).

Vg is present in the hemolymph of all female caste members of the red imported fire ant, *Solenopsis invicta*, including workers, alate (virgin) females and mated queens (Lewis et al., 2001). Vg titers are comparable in mated queens and alate females, although no egg maturation is observed in alate females (Vargo and Laurel, 1994; Lewis et al., 2001). This suggests that the Vg gene is active constitutively, and regulations of yolk formation could perhaps occur at the level of Vg uptake into the oocyte, rather than at the level of Vg synthesis by the fat body. Hence, understanding VgR biology may be key to understanding imported fire ant queen reproduction.

The *S. invicta* VgR (*Si*VgR) cDNA cloning, sequencing and ensuing studies are presented in chapters, each covering a different aspect of the project. Chapter III focuses on isolation of *Si*VgR cDNA leading to Chapters IV and V. Chapter IV presents the tissue- and caste-specific transcriptional expression of *Si*VgR. Chapter V includes the *Si*VgR transcriptional expression timing profiles in virgin alate females and newly mated queens, and contains the study of the endocrine regulation of *Si*VgR transcriptional expression *in vitro*.

CHAPTER II

LITERATURE REVIEW: THE LOW-DENSITY LIPOPROTEIN RECEPTOR SUPERFAMILY

Lipid transport via the circulatory system of animals constitutes a vital function that generally requires lipoprotein complexes. Lipoproteins have been studied extensively in vertebrates, especially mammals. Vertebrates rely on so-called high- and low-density lipoproteins to transport lipid in blood. Insect hemolymph generally contains a single major lipoprotein, lipophorin, which is found in relatively large quantities (Ryan, 1990). A characteristic feature of lipophorin is an ability to function as a reusable lipid shuttle by the selective loading and unloading of lipids at different target tissue sites (Ryan, 1990; Van der Horst, 1990). The major lipid components of insect lipophorins are diacylglycerides (DAG) and phospholipids. As a function of physiological or developmental needs for lipid distribution, lipophorin may exist in several forms with respect to relative lipid content and apolipophorin composition, leading to differences in size and density of the particle. The apolipophorin is the protein component of lipophorin. The apolipophorin is synthesized in the fat body where it is combined with phospholipids and released into the hemolymph as nascent lipophorin particles. High-density lipophorin (HDLp) contains relatively little lipid; low-density lipophorin (LDLp) contains more lipids. HDLp and LDLp are not discrete categories. During metabolism, addition of lipid to HDLp converts it to LDLp while only small changes occur in the volume and density of the particle (Ryan, 1994).

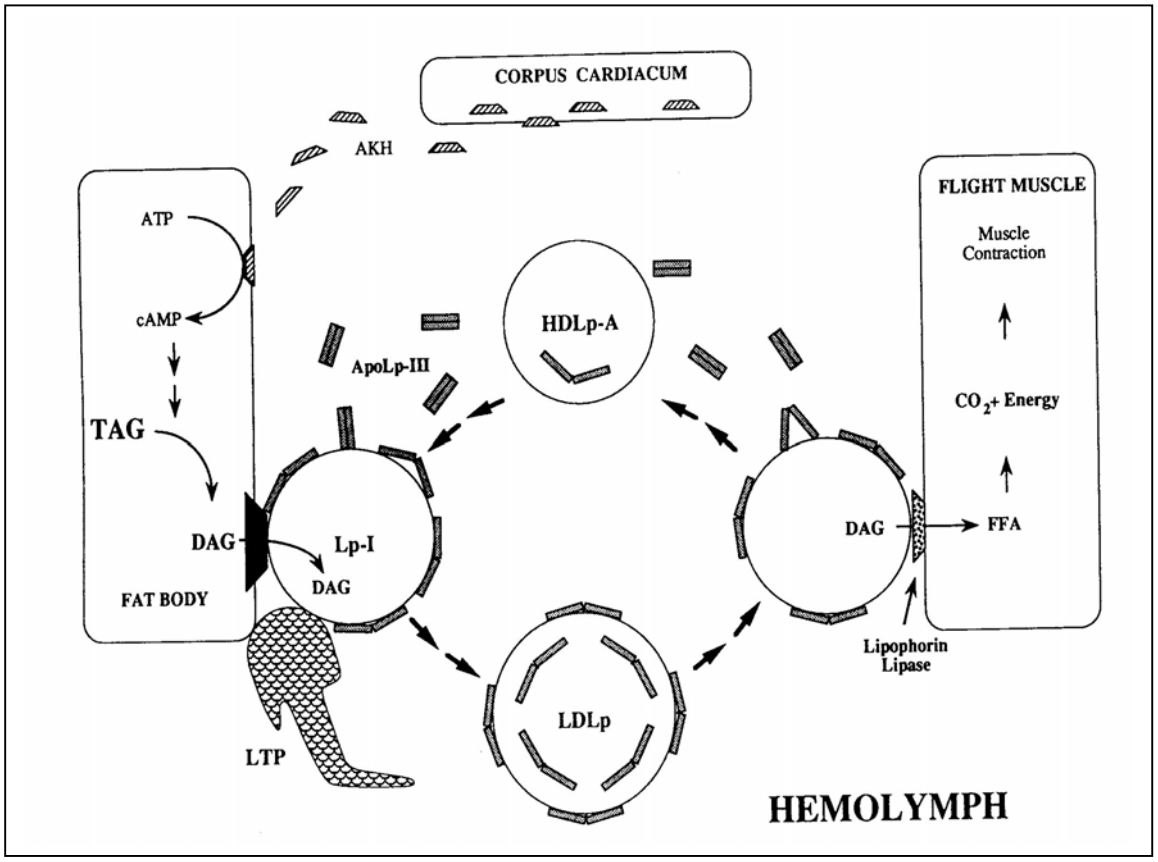

Fig. 2-1. Model of lipid mobilization and transport by lipophorin in insects which rely on lipid as their principle flight energy source. AKH: adipokinetic hormone; TAG: triacylglycerol; DAG: diacylglycerol; ApoLp-III: apolipophorin III; HDLp-A: high-density lipophorin-adult; LDLp: low-density lipophorin; Lp-I, lipophorin loading intermediate; FFA: free fatty acid; LTR: lipid transfer particle (Ryan, 1990).

Lipophorin transports lipid from the midgut to the fat body, from the fat body to the flight muscles and also delivers lipids to the developing oocytes (Blacklock and Ryan, 1994). Flight-induced lipid loading (Fig. 2-1) onto HDLp is accompanied by association of multiple copies of the exchangeable apolipophorin III (ApoLp-III), which serve to stabilize the lipids and modulate metabolism of the lipophorin particle. Adipokinetic hormone (AKH) is released from the corpus cardiacum and binds to receptors in the fat body, where it activates lipolysis of triacylglycerol (TAG) to diacylglycerol (DAG).

The DAG leaves the fat body with the assistance of a lipid transfer particle (LTP) and is taken up by HDLp-A to form the lipophorin-loading intermediate (Lp-I). The capacity of HDLp-A to carry DAG is increased by binding ApoLp-III to its surface. Ultimately, LDLp is formed and carries the DAG to the flight muscle, where a lipophorin lipase hydrolyzes the DAG to produce free fatty acids and regenerates HDLp-A and ApoLp-III. The free fatty acids enter the flight muscle and are oxidized to produce the ATP required to power flight. HDLp-A and ApoLp-III circulate back to the fat body to repeat the cycle (Ryan, 1990). In this way, lipophorins are essential for insect energy metabolism when lipids are the main source of flight fuel.

Lipophorins are also important for insect reproduction, and employ endocytosis to transmit the lipid to the oocyte. HDLp-A (high-density lipophorin-adult) is taken up selectively by the developing oocyte. Within the ooctye, HDLp-A is converted to VHDL-E (very high-density lipophorin-egg) due to lipid unloading (Telfer et al., 1991). Also vitellogenin (Vg), the major egg yolk protein precursor, is a lipophorin and is taken up by the oocyte through receptor-mediated endocytosis as shown in several insects (Ferenz and Lubzens, 1981; Osir and Law, 1986; Oliveira et al., 1986; Kulakosky and Telfer, 1987; Kindle et al., 1988; Konig et al., 1988; Koller et al., 1989).

1. CHARACTERISTICS AND STRUCTURAL FEATURES OF THE LOW-DENSITY LIPOPROTEIN RECEPTORS (LDLR)

The LDLRs share structural and functional properties and interact with a diverse group of ligands. There are several characteristic features of the LDLR superfamily

(Hussain et al., 1999; Schneider et al., 1999): cell surface expression; an extracellular ligand binding domain consisting of Class A cysteine-rich repeats (also called ligand-binding or complement-type repeats); a requirement of Ca^{2+} for ligand binding; recognition of receptor-associated protein (RAP); an epidermal growth factor (EGF) precursor homology domain containing Class B cysteine-rich repeats (also called EGF repeats) and YWXD amino acid repeats; a single membrane-spanning region; receptor-mediated endocytosis of various ligands, and the fact that the structurally and functionally distinct modules are often defined by distinct exons in the corresponding genes.

The LDLRs are highly conserved consisting of five domains after cleavage of the signal sequence (Fig. 2-2). These domains are: 1) the ligand binding domain, 2) the EGF precursor homology domain, 3) an O-linked carbohydrate domain, 4) a transmembrane domain, 5) a cytoplasmic tail (Goldstein et al., 1985; Schneider, 1996).

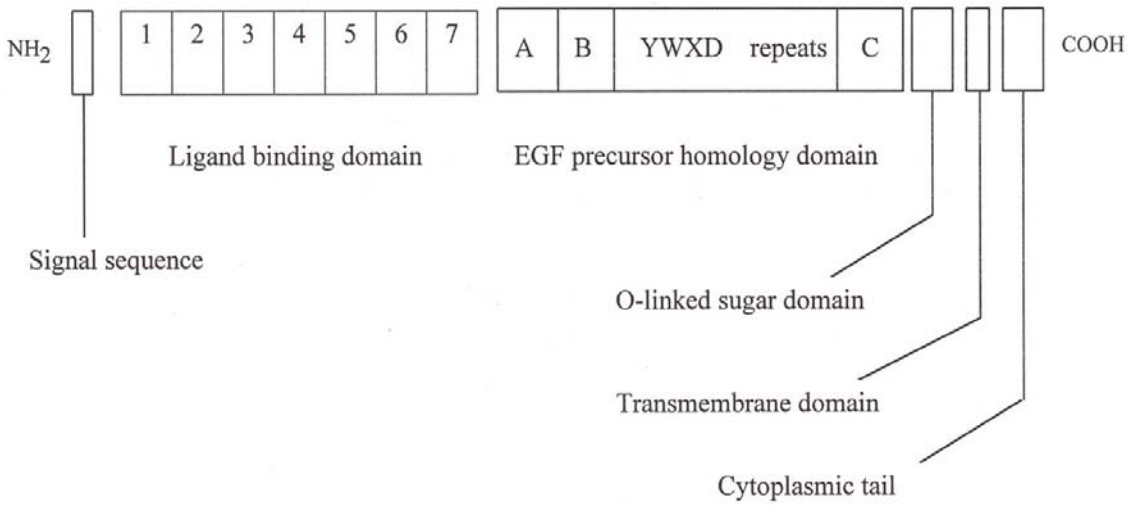

Fig. 2-2. The arrangement of protein domains in the prototype receptor, the human LDLR. 1-7: Class A cysteine-rich repeat. A, B and C: Class B cysteine-rich repeat.

In LDLR superfamily, there are two types of cysteine-rich repeats, Class A and Class B, respectively. The differences between these two classes are based on the disulphide bond structure and the presence or absence of the negatively-charged amino acid sequence (SDE).

1.1. The ligand binding domain

It consists of Class A cysteine-rich repeats of about 40 amino acids each. Each repeat contains six cysteine residues which are disulphide bonded in the pattern one to three, two to five and four to six (Bieri et al., 1995). Reduction of these disulphide bonds destroys the structure and abolishes binding activity (Daniel et al., 1983). A striking feature of the C-terminus of each repeat sequence is a cluster of negatively-charged amino acids (SDE). These sequences are putatively complementary to positively-charged sequences distributed on the surface of the ligand (Goldstein et al., 1985). The intriguing fact is that based on nuclear magnetic resonance (NMR) studies (Daly et al., 1995) and crystallographic studies (Fass et al., 1997) the negatively-charged residues are buried in the core coordinated with Ca^{2+}. Thus the binding activity may be contributed by the overall three-dimensional structure. For human LDLR, the repeat 5 is required for binding of apolipoprotein E (apoE), and repeats 3-7 cooperatively bind to apoB (Schneider, 1996). Both ligands differ in amino acid sequence and have no known structural relationship other than their common affinity for negatively charged polymers. So different combinations of repeats allow a single receptor to recognize structurally diverse ligands (Brown et al., 1997).

1.2. The EGF precursor homology domain

The EGF precursor homology domain consists of Class B cysteine-rich repeats and YWXD repeats. Compared to Class A cysteine-rich repeats, the Class B cysteine-rich repeats in this domain are arranged in a different disulphide bond pattern as follows: one to three, two to four and five to six (Campbell and Bork, 1993), and they also lack the negatively-charged residues (SDE) at the C-terminus. The Class B cysteine-rich repeats are usually separated by the YWXD repeats that are found as a group of five in a module of about 50 amino acids (Hussain et al., 1999) (Fig. 2-2). Within endosomes, following endocytosis, this domain contributes to ligand dissociation from the receptors. When the EGF precursor domain is deleted from the LDLR, the receptor can not bind LDL, but it still binds lipoproteins that contain apoE. The bound apoE enter the cell normally, but they are not released in the endosome. Instead, the receptor is degraded along with the ligand (Davis et al., 1987).

1.3. An O-linked sugar domain

The O-linked sugar domain of the human LDLR is highly enriched in serine and threonine residues and located just outside the plasma membrane (Yamamoto et al., 1984). Deletion of this domain in the LDLR does not affect ligand binding, internalization, recycling, or receptor half-life in cultured cells (Davis et al., 1986). It suggests that this domain is not required for receptor function.

1.4. A transmembrane domain

All the members of the LDLR superfamily contain a single transmembrane domain consisting of a stretch of hydrophobic residues that anchors them into membranes. The sequence of this domain is the least conserved of all the receptor domains among seven mammalian species (Schneider, 1996). Comparison of the amino acid sequences of the mosquito and fruit fly VgRs also reveals that the membrane-spanning region is poorly conserved (Sappington et al., 1996).

1.5. A cytoplasmic tail

Compared to signaling receptors, which often contain large intracellular domains with kinase activities, the cytoplasmic tails of the LDLR superfamily members are relatively short with no kinase domain. The cytoplasmic domain of the LDLR plays an important role in clustering coated pits, either through interaction with clathrin itself or with some protein associated with clathrin on the cytoplasmic side of the membrane (Goldstein et al., 1985). The internalization signal, NPXY, is identified within the LDLR (Chen et al., 1990). The di-leucine motif is another well-known internalization signal that is present within many transmembrane cell surface proteins (Letourneur and Klausner, 1992; Dittrich et al., 1994). For insect vitellogenin receptors (VgRs), mosquito VgR contains one leucine-isoleucine, and fruit fly VgR contains two di-leucine motifs in the cytoplasmic tails as internationalization signal (Sappington et al., 1996). However, recent studies indicate that in addition to the internalization signals, the cytoplasmic tails of LDLRs contain critical elements for interaction with a set of

cytoplasmic adaptors and scaffold proteins, and mediate signal transduction (Strickland et al., 2002).

2. THE LDLR SUPERFAMILY MEMBERS

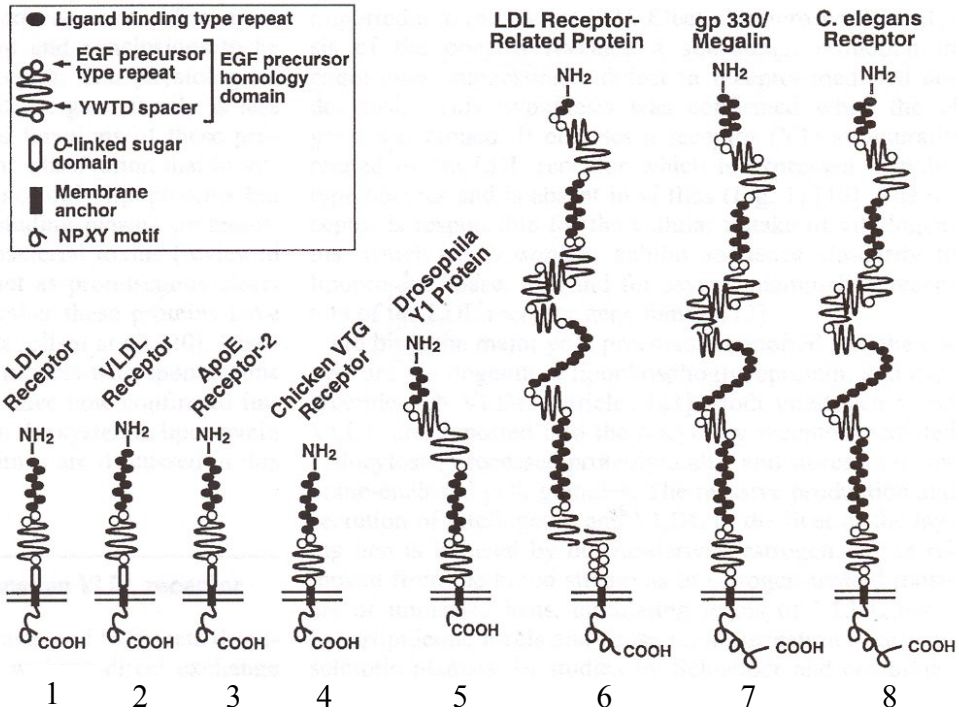

Fig. 2-3. The members of LDLR superfamily (modified after Willnow, 1999).

2.1. The human LDLR

Since 1974, the discovery of a cellular pathway for the binding, internalization and degradation of plasma low-density lipoprotein (LDL) led to the identification of the human LDLR (Goldstein and Brown, 1974; Yamamoto et al., 1984). LDLRs are under intense investigation because of their importance in human heart disease. The human

LDLR gene is more than 45 kb in length and contains eighteen exons, most of which correlate with functional domains (Sudhof et al., 1985). The nucleotide sequence of a cloned 5.3 kb cDNA for the human LDLR reveals five domains in the 839 amino acid protein (Yamamoto et al., 1984). The N-terminal ligand binding domain is characterized by the presence of seven Class A cysteine-rich repeats also designated as ligand binding type repeats (Fig. 2-3). See number 1. The major function of the LDLR is cholesterol homeostasis, which is accomplished by controlling plasma LDL levels. Mutations in the LDLR gene cause familial hypercholesterolemia characterized by increased plasma cholesterol levels, arcus cornea, tendon xanthomas, and coronary heart disease (Goldstein et al., 1995). Four classes of mutations that disrupt the structure and function of the LDLR cause familial hypercholesterolemia (Goldstein et al., 1985). Class 1 mutations: no receptors synthesized. This is the most common class of mutant alleles. The genetic defects in the LDLR may include nonsense mutations; mutations in the promoter that block transcription of mRNA; point mutations in intron-exon junctions that alter the splicing of mRNA and large deletions. Class 2 mutations: receptors synthesized but transported slowly from ER to Golgi. Class 3 mutations: receptors are processed and reach the cell surface, but fail to bind LDL normally. Class 4 mutations: receptors reach cell surface and bind LDL, but fail to cluster in coated pits. The failure of these internalization defective receptors to localize to coated structures results from mutations that directly or indirectly disrupt the cytoplasmic tail of the receptor, in particular, the sequence FDNPXY (Lehrman et al., 1985).

Although most of the mutations map in the coding region of the gene, an increasing number of mutations in the regulatory regions are currently being described (Peeters et

al, 1998; Scholtz et al., 1999; Mozas et al., 2002). The basic regulatory region of the LDLR gene is located within 177 bp of the proximal promoter. The 177-bp fragment of the receptor gene contains all of the detectable signals for positive expression as well as for negative regulation by sterols. This fragment contains the three direct repeats, each of which is required for mRNA expression. It also contains the two TATA-like sequences, only one of which is required for expression (Sudhof et al., 1987b).

The direct repeats are homologous to the consensus sequence recognized by the transcription factor Sp1 (Sudhof et al., 1987b). Repeats 1 and 3 contain binding sites for the transcription factor Sp1 and contribute to the basal expression of the LDLR gene. DNase I footprint analysis indicates that the mutation, a 3-bp deletion in repeat 1, abolished binding of Sp1 to repeat 1. Transcription studies in transfected cells using normal and repeat 1 mutant promoter fragments linked to a luciferase reporter gene demonstrate that the repeat 1 mutant promoter has approximately 10% of normal promoter activity (Peeters et al., 1998). Specific binding of purified Sp1 to repeat 3 was demonstrated by electrophoretic mobility shift assays (EMSA) and DNase I footprinting assays (Dawson et al., 1988).

Sterol-dependent repression of transcription is mediated by a 42-bp region encompassing repeats 2 and 3 (Sudhof et al., 1987a). Repeat 2 harbors a special element that is directly responsible for regulation by sterols and does not bind Sp1. The sterol regulatory element (SRE-1) within repeat 2 was identified by mutational studies as a 10-bp sequence that binds a family of proteins called the sterol regulatory element binding proteins (SREBPs) (Briggs et al., 1993). SRE-1 functions as a conditionally positive element activating expression only when sterol levels are low (Briggs et al.,

1993). SREBPs binding at repeat 2 increased the binding of Sp1 to the adjacent repeat 3. The Sp1 has a dual role in the LDLR promoter. It provides a basal activation required for a low suppressed level of promoter activity, and it also participates in sterol regulation of the LDLR. Its role in sterol regulation requires that it interact with SREBP bound to the adjacent repeat 2 (Sanchez et al., 1995).

In addition to sterol, LDLR transcription can also be regulated by nonsterol mediators. Cytokine oncostatin M (OM) is a growth regulatory protein secreted by macrophages and activated by T lymphocytes. OM increases the expression of LDLR protein and mRNA independent of intracellular cholesterol (Grove et al., 1991). To elucidate regulatory mechanisms, specific mutants in repeats 1, 2, and 3 were made to facilitate the mapping of the OM effect on the promoter. The mutation within the SRE-1 element of repeat 2 totally abolished cholesterol regulation but had no effect on OM inducibility. Instead, mutations within repeats 1 and 3 impaired OM activity (Liu et al., 1997). The OM-responsive element to promoter region contains the repeat 3 and two TATA-like sequences (Li et al., 1999a). Within the two TATA-like promoter regions, a sterol-independent regulatory element (SIRE) was designated. The 17-bp-SIRE contains an active CCAAT/enhancer-binding protein (C/EBP)-binding site and a functional cAMP-responsive element (CRE) (Liu et al., 2000). Electrophoretic mobility shift assays confirm the specific binding of transcription factors C/EBP and cAMP-responsive element-binding protein to the SIRE. Mutations within either the C/EBP or CRE site have no impact on basal or cholesterol-mediated repression of LDLR transcription, but they completely abolish OM-mediated activation of LDLR transcription (Liu et al., 2000).

2.2. Very-low-density lipoprotein receptor (VLDLR)

2.2.1 Vertebrate

The structure of the VLDLR is similar to the LDLR (Fig. 2-3). See number 2. The major difference is that there is an additional Class A cysteine-rich repeat containing in the VLDLR ligand binding domain (Sakai et al., 1994). Both cDNA and the gene structures of these two receptors are similar. The different functional motifs in the VLDLR are also encoded by different exons as observed in the LDLR (Sakai et al., 1994). However, the tissue distribution of the VLDLR is different from that of the LDLR. The VLDLR is not present in liver but is abundant in skeletal muscle, heart, adipose tissue and brain (Takahashi et al., 1992; Webb et al., 1994). The VLDLR was originally thought to play a role in triglyceride metabolism, but the triglyceride, total plasma cholesterol, and free fatty acid levels of VLDLR homozygous knockout mice are indistinguishable from wild type controls (Frykman et al., 1995). It is because of overlapping ligands with other LDLR family members. The role for VLDLR in triglyceride metabolism is revealed by cross breeding VLDLR-deficient mice and VLDLR-over-expressing mice with LDLR homozygous knockout mice. On the LDLR deficient background, absence of the VLDLR results in a significant increase in serum triglyceride levels when mice are fed a high fat diet. In contrast, overexpression of the VLDLR results in a significant decrease of serum triglyceride levels under similar conditions (Tacken et al., 2000). These observations indicate that the VLDLR affects peripheral uptake of triglyceride.

The VLDLR shows a high degree of identity among different species. Within mammals, there is a 95% identity between the corresponding proteins. In addition, even

the proteins of more distant species such as the chicken and *Xenopus laevis* share 84% and 73% identity respectively with the human VLDLR (Schneider et al., 1999).

2.2.2 Invertebrate

In the locust, *L. migratoria*, a high-affinity HDLp binding site ($K_d \sim 10^{-7}$ M) has been characterized in intact fat body tissue as well as in fat body membranes of larval and adult locusts (Van Antwerpen et al., 1989; Dantuma et al., 1996). The lipoprotein binding site was identified as an endocytotic receptor involved in receptor-mediated uptake of HDLp. The putative receptor involved in endocyteic uptake of HDLp in the locust fat body has been cloned and sequenced (Dantuma et al., 1999). This receptor contains eight cysteine-rich repeats in its putative ligand-binding domain like the vertebrate VLDLR does. This receptor shows high similarity to rabbit and human VLDLR, 58.6% and 58.0%, respectively. Northern blot analysis revealed one transcript of approximately 4.8 kb in locust fat body, oocytes, brain and midgut, but the transcript was absent from flight muscle (Dantuma et al., 1999). Expression of the receptor mRNA in fat body cells is down-regulated during adult development, which is consistent with the down-regulation of receptor-mediated endocytosis of lipophorins in fat body tissue (Dantuma et al., 1997, 1999). The function of receptor-mediated endocytosis remains unclear because inhibition of endocytosis did not reduce the exchange of DAG or cholesterol between HDLp and the fat body cell (Dantuma et al., 1997).

2.3. ApoE receptor 2

Apolipoprotein E (ApoE)-mediated lipid metabolism in the central nervous system plays an important role in cholesterol and phospholipid homeostasis of this system. ApoE receptor 2, or LR7/8B, was cloned from brain cDNA libraries of human, mice, and chickens (Kim et al., 1996; Novak et al., 1996). The human receptor contains seven ligand binding repeats, whereas mouse and chicken receptors contain eight ligand binding repeats. The variants observed in different species probably arise because of differential splicing (Brandes et al., 1997). The EGF precursor homology domain, an O-linked glycosylation motif, a transmembrane region and the cytoplasmic domains are similar to those of the LDLR and VLDLR (Fig. 2-3). See number 3. With respect to sequence homology and restricted tissue expression, the apoE receptor 2 is closer to VLDLR than to LDLR. Thus, it is expected to have broad ligand binding ability, similar to that observed for VLDLR, rather than the restricted specificity observed for vertebrate LDLR.

2.4. Vertebrate Vg receptor

2.4.1 Chicken Vg receptor

An interesting VLDLR is the chicken VgR relative to its role in the reproductive effort of the hen. This receptor is termed LR8 with eight Class A cysteine-rich repeats in the ligand binding domain (Bujo et al., 1994) (Fig. 2-3). See number 4. Splice variant forms of the chicken LR8 gene produce an oocyte-specific VLDLR/VgR without an O-linked sugar domain or a soma-specific variant containing an O-linked domain (Bujo et al., 1995a). The functional absence of LR8 leads to female sterility via lack of oocyte

growth and premature atherogenesis due to the severe hyperlipidemia (Bujo et al., 1995b). During vitellogenesis the oocytes accumulate large amounts of macro- and micronutrients required for the embryo. In chickens, triglyceride-rich, VLDLs and Vg comprise the major part of yolk (Perry and Gilbert, 1979). The single major chicken oocyte plasma membrane receptor, VLDLR/VgR has a molecular mass of 95 kDa as determined by SDS-PAGE under nonreducing conditions and is the receptor for both of these ligands (Stifani et al., 1990). The isolated receptor had the capacity to bind ^{125}I-VLDL and ^{125}I-Vg with high affinity and in saturable fashion when analyzed by a solid phase filtration assay (Barber et al., 1991). This receptor reacts with antibodies to mammalian LDLR and surprisingly recognizes apoE, which is not produced in birds (Steyrer et al., 1990). However Vg and apoE have certain common biochemical properties and regions of sequence similarities and have been suggested to be functional analogs (Steyrer et al., 1990). These properties predicted that the oocyte receptor for VLDL and Vg would be a homolog of mammalian LDLR.

2.4.2. Frog Vg receptor

During *Xenopus laevis* oocyte growth, 80–90 % of total yolk protein is derived from the specific uptake of Vg from the maternal blood stream (Wallace et al., 1972). The rate of Vg uptake in oocytes regulates overall oocyte growth and differentiation and can be modulated by gonadotropins and insulin (Wallace and Misulovin, 1978; Wiley and Dumont, 1978). *Xenopus* VgR is probably developmentally regulated, since neither stage I nor II oocytes appear to be able to incorporate Vg. It is quite possible that these cells do not possess VgRs at this stage of development (Dumont, 1972). A *Xenopus* 3.6

kb cDNA clone contains the entire open reading frame and 5' and 3' noncoding regions. The deduced amino acid sequence is 72 % homologous to the chicken VLDLR/VgR and the characteristic domains are highly conserved (Okabayashi et al., 1996). The *Xenopus* VgR has eight Class A cysteine-rich repeats which is characteristically conserved among the VLDLR and vertebrate VgR family. In addition, the amino acid sequence of *Xenopus* is more similar to the chicken VLDLR/VgR sequence (72% identity) than to the *Xenopus* LDLR sequence (44% identity) (Mehta et al., 1991; Okabayashi et al., 1996). Radiolabeled *Xenopus* Vg binds to the membrane VLDLR/VgR in the chicken oocyte. Similarly only one 115 kDa membrane protein from *Xenopus* oocytes interacted with ^{125}I-labeled *Xenopus* Vg (Stifani et al., 1990). The binding of radiolabeled Vg to both chicken and *Xenopus* oocyte membrane proteins is totally abolished when membrane proteins are exposed to reducing agents for disulfide-bonds. This agrees with the fact that intrachain disulfide bonds within the receptor molecule are necessary for retention of receptor activity (Stifani et al., 1990; Barber et al., 1991).

2.4.3. Fish Vg receptor

The VgR of the coho salmon (*Oncorhynchus kisutch*) was characterized from oocytes. In direct binding studies, the receptor exhibits high affinity for salmonid Vg (K_d, 180 nM) and binding is saturable. Ligand blotting with radiolabeled Vg under nonreducing conditions, reveal a protein with an apparent Mr. of 100 kDa. The piscine receptor showed cross-reactivity with the chicken and frog receptors both in terms of

ligand recognition and immunoreactivity (Stifani et al., 1990). Salmon VgR shares key structural elements with VgRs from chicken and *Xenopus*.

In the rainbow trout, *Oncorhynchus mykiss*, Vg uptake constitutes > 80% of the growth of the oocyte and accounts for most of the 1000-fold increase in size that takes place during vitellogenesis (Tyler and Sumpter, 1996). Studies on VgR protein in the rainbow trout have shown that it is present in oocytes throughout the major part of ovarian development, and that it can be detected even before the onset of vitellogenesis (Lancaster and Tyler, 1994). The expression of VgR mRNA varied throughout ovarian development and is highest in previtellogenic ovaries and in ovaries at the onset of vitellogenesis containing ovarian follicles from 35 to 600 μm in diameter (Perazzolo et al., 1999).

Tyler and Lubberink (1996) showed by ligand blotting that rainbow trout ovarian follicles contained four receptor proteins of Mrs. 220, 210, 110 and 100 kDa, respectively. These receptors have a high affinity for Vg but did not appear to bind other homologous plasma lipoproteins (VLDL, LDL and HDL). Furthermore, these binding sites for Vg were specific to the ovary. At that stage, the authors cannot say whether the receptor for Vg in the rainbow trout is comprised of two associated ~100 kDa proteins or forms dimeric structures on its removal from the membrane. In the same year, Nunez Rodriguez *et al*. (1996) reported that the ligand blotting with ^{125}I-Vg after SDS-PAGE revealed the existence of one major binding component corresponding to a protein of 113 kDa.

A rainbow trout oocyte-specific receptor cDNA has been cloned (Davail et al., 1998). *In vitro* translation of the full-length cDNA produces a 97 kDa protein and

transient expression in mammalian COS-1 cells showed that the cDNA encodes a protein of the same size that binds Vg in ligand blots (Davail et al., 1998). The cloned VgR, a 826-residue type I membrane protein, is a member of the LDLR superfamily. It closely resembles the mammalian VLDLR, in that its N-terminal ligand binding domain consists of a cluster of eight Class A cysteine-rich repeats. The short cytoplasmic tail contains the internalization signal, FDNPVY, typical for the LDLR superfamily. Notably, the receptor lacks a domain with a high density of potential O-glycosylation sites often found in somatic cell-specific members of the LDLR family. A transcript of 3.9 kb is abundant in ovary, but undetectable in muscle and heart, which are the major sites of expression of VLDLRs in mammals (Davail, et al., 1998).

In the rainbow trout ovary, cDNA encoding for a second lipoprotein receptor has been partially characterized from the ovary, which differs only in sequence from the VgR by an additional 105 base pairs that constitutes the O-linked sugar domain (Prat et al., 1998). This receptor has been referred to as the somatic lipoprotein receptor, and it is expressed both in the ovary and in somatic tissues. The precise function of the somatic lipoprotein receptor is not known, but the ovary lipoprotein receptor is thought to mediate the uptake of lipoproteins other than Vg, for example, VLDL that could be a component of yolk proteins (Prat et al., 1998).

2.5. Invetebrate vitellogenins and vitellogenin receptors (VgR)

2.5.1. Insect Vgs

Like all oviparous animals, insects provision their eggs with proteins, lipids, carbohydrates and other resources for the sustenance of the developing embryo. Insect

Vg is synthesized extraovarially and becomes the major egg yolk protein. Vgs are large (200-700 kDa) phosphoglycolipoproteins that are often oligomeric in their native state with monomers consisting of one to four subunits. The Vg monomers of most insects are composed of one large (> 150 kDa) and one small (< 65 kDa) subunit (Raikhel and Dhadialla, 1992). Although the primary function of Vg is to provide a pool of amino acids for the embryo, it also functions as a carrier of carbohydrates, lipids, phosphates, vitamins, metals, and hormones. Such transport functions, along with the necessity for specifically binding a VgR, presumably place constraints on Vg structure (Sappington and Raikhel, 1998). The most striking feature of vertebrate and some insect Vg primary structures are the presence of one to three domains containing long runs of serine residues. The serine residues probably serve as substrates for kinases. Phosphoserine tracts represent extreme concentrations of negative charge, which may promote solubility of the Vg or provide a region for chelating essential metal ions such as Ca^{2+} and Fe^{3+} (Sappington and Raikhel, 1998). Dephosphorylation of Vg reduces its uptake by oocytes, suggesting a role of phosphorylated residues in VgR recognition or maintenance of tertiary structure (Dhadialla et al., 1992).

Studies on binding of Vg to membranes isolated from follicles or ovaries have been made on a few insect species: *L. migratoria* (Rohrkasten and Ferenz, 1986a), *N. cinerea* (Konig and Lanzrein, 1985), *M. sexta* (Osir and Law, 1986), *B. germanica* (Konig et al., 1988), *R. prolixus* (Wang and Davey, 1992), and *A. aegypti* (Dhadialla and Raikhel, 1991). In all these studies, binding of Vg was characteristic of a receptor-ligand interaction in which it was saturable, ovary specific, selective for Vg and sensitive to changes in pH and Ca^{2+} concentration. In *Rhodnius*, binding of vitellin (Vn) is

enhanced by JH-I, which appears to increase the number of receptors (Wang and Davey, 1992). Also an increase in Vg uptake by ovaries *in vitro* occurs in *N. cinerea* in the presence of JH-III (Kindle et al., 1988). On the other hand, the JH analog, ZR515, is without effect on endocytosis in locusts (Rohrkasten and Ferenz, 1985). Solubilization of VgRs has been achieved for *L. migratoria* (Rohrkasten and Ferenz, 1986b), *N. cinerea* (Indrasith et al., 1990) and *A. aegypti* (Dhadialla et al., 1992).

2.5.2. *Drosophila* vitellogenin receptor, Yl protein

To date, VgR cDNAs have been sequenced in three insects, the mosquito, *A. aegypti*, fruit fly, *D. melanogaster*, (Schonbaum et al., 1995; Sappington et al., 1996) and American cockroach, *Periplaneta americana* (accession number BAC 02725). In the fruit fly, an ovary-specific, 6.5 kb yolkless (*yl*) mRNA encoding a protein of ~210 kDa was identified by a combination of complementation mapping, chromosome walking, and northern blotting (Schonbaum et al., 1995). Sterile mutant female fruit flies carrying the *yl* gene fail to accumulate yolk protein in their oocytes (Waring et al., 1983; Perrimon, et al., 1986). When a genomic DNA fragment encompassing this 6.5 kb transcript was introduced into *yl* flies, yolk uptake and fertility were restored (Schonbaum et al., 1995).

yl RNA and protein are both expressed very early during the development of the oocytes, long before vitellogenesis begins. RNA *in situ* hybridization and *lacZ* reporter analyses show that *yl* RNA is synthesized by the germ-line nurse cells and then transported to the oocyte. Yl protein is evenly distributed throughout the oocyte during the previtellogenic stages. The transition to the vitellogenic stages is marked by the

accumulation of yolk via clathrin-coated vesicles. After this transition, VgR levels increase markedly at the cortex of the egg (Schonbaum et al., 2000).

The translation product of the 5844-nt open reading frame encoded for by the *yl* cDNA is a 1984-residue protein that contains several repeats of the structural elements that typify the LDLR superfamily (Fig. 2-3). See number 5. There are thirteen Class A cysteine-rich repeats in two clusters of five and eight repeats each, respectively; seven Class B cysteine-rich repeats that flank three groups of five YWXD repeats each and a single membrane-spanning domain. In contrast to the extracellular domain, the putative cytoplasmic domain of Yl shows no similarity to other LDLR superfamily members. Most conspicuous is the absence of the NPXY sequence common to other LDLR proteins. However, the Yl cytoplasmic tail does have two di-leucine motifs that are identified as alternative internalization signals (Letourneur and Klausner, 1992; Dittrich et al., 1994). Yl does not appear to be more closely related to the chicken VgR than to other LDLR superfamily members. A sequence comparison of the eight-repeat clusters in the chicken VLDLR/VgR and *Drosophila* Yl reveals 38% amino acid identity; while between rabbit and chicken VLDLR, there is 84% amino acid identity in this region (Bujo et al., 1994; Schonbaum et al., 1995).

Homology of VgRs and LDLRs has also been suggested by the similarity of their ligands, since Vgs from a broad range of species (e.g., birds, amphibia, and nematodes) have regions of sequence similarity to apoB-100, which is a ligand for LDLR (Baker, 1988). However, the yolk proteins of higher dipterans, such as *Drosophila*, *Ceratitis*, and *Calliphora* show similarity to lipoprotein lipase of higher animals, which are known to bind to LDLR-related proteins (LRP) (Terpstra and Ab, 1988; Beisiegel et al.,

1989; Rina and Savakis, 1991; Martinez and Bownes, 1994). In chickens, a ~380 kDa oocyte-specific LRP as well as the 95-kDa VgR bind Vg (Stifani et al., 1991). Therefore more than one receptor of the LDLR superfamily may be responsible for the coordinated uptake of individual or structurally similar groups of yolk precursors.

2.5.3. Mosquito *Aedes aegypti* ovary receptors

The *A. aegypti* VgR (*Aa*VgR), is identified as a 205-kDa protein by ligand blotting under nonreducing conditions (Dhadialla et al., 1992). Native gel analysis suggests that it occurs as a non-covalent homodimer (Sappington et al., 1995).

The VgR is present only in ovarian tissue and immunocytochemical experiments revealed that it is present only in the oocyte, not in follicle cells or nurse cells (Dhadialla et al., 1992; Sappington et al., 1995). The full length *Aa*VgR cDNA, 5544 bp, has been cloned (Sappington et al., 1996). The 7.3 kb transcript is present in oocytes and nurse cells of primary follicles and germ-line cells of the germarium as determined by northern blot analysis and *in situ* hybridization experiments. The level of VgR transcript starts to rise in the ovary one day post-eclosion. It continues to rise during the previtellogenic and vitellogenic period and reaches its peak at 24 h post-blood meal, suggesting the *Aa*VgR gene is expressed early in oocyte differentiation (Sappington et al., 1996; Cho and Raikhel, 2001).

Analyses of the *Aa*VgR deduced amino acid sequence indicate that it is a member of the LDLR superfamily. The modular arrangement is similar to that of *Drosophila* VgR except that *Aa*VgR contains an O-linked sugar domain between the final extracellular cysteine residue in the Class B cysteine-rich repeats and the beginning of the

transmembrane domain. The cytoplasmic tail of *Aa*VgR lacks the conserved internalization signal, NPXY. Instead, it contains a leucine-isoleucine motif that is identified as an alternative internalization signal. The striking homology of the *Aa*VgR to the *Drosophila* VgR (42 % amino acid identity and 63 % similarity) is very surprising since the primary structures of their ligands are unrelated (Sappington et al., 1996).

The *Aa*VgR gene structure has been determined (Cho and Raikhel, 2001). The VgR gene is separated by five introns that have an average length of 60 bp, except for the second intron, which is more than 20 kb long. The exon-intron organization of the *Aa*VgR is quite different from other cloned genes of the LDLR superfamily. The introns of *Aa*VgR are present in the N-terminal half, which contains the first ligand binding domain and the first EGF precursor homology domain (Cho and Raikhel, 2001). Nevertheless, nineteen introns of the human LDLR (Sudhof et al., 1985), VLDLR (Sakai et al., 1994) and eighteen introns of mouse VLDLR (Tiebel et al., 1999) interrupt the coding sequence all along different domains, such as the ligand binding domain, the EGF precursor homologous domain, the O-linked sugar domain, the transmembrane domain and the cytoplasmic domain. However, most of the introns interrupt the coding sequences at or close to boundaries between domains and between units within a domain. Sudhof *et al.* (1985) suggested that the presence of numerous introns at boundaries of Class A cysteine-rich repeats of the human LDLR gene could lead to the splicing of mRNAs coding for receptors with different ligand-binding characteristics.

Although Vgs are lipidated and oocytes internalize large amounts of Vgs, the amount of lipid associated with these proteins is far less than the total lipid in insect

eggs. Another class of proteins, lipophorins (Lp), carry lipid to various tissues, including the eggs (Raikhel and Dhadialla, 1992). In lepidopteran vitellogenesis, Lp, the major lipoprotein in insect hemolymph, plays a dual role. It shuttles precursors from the fat body to the ovaries for the deposition of lipid yolk droplets, and in some species becomes one of the major constituents of the protein yolk bodies (Telfer et al., 1991). Lp functions as a yolk protein precursor in the mosquito *A. aegypti* and it is internalized via receptor-mediated endocytosis (Sun et al., 2000). A putative mosquito lipophorin receptor from ovaries (*Aa*LpRov) cDNA has been cloned (Cheon et al., 2001). The *Aa*LpRov cDNA has a length of 3468 bp coding for a 1156-residue protein with a predicted Mr. of 128.9 kDa. *In situ* hybridization and northern blot analysis indicate that the 4.5 kb transcripts are present only in female germ line cells. The *Aa*LpRov transcripts are present in the previtellogenic stage and increase further after the onset of vitellogenesis with peak expression by 24 h post-blood meal. It is present in the ovary until 48 h post-blood meal, the time of termination of vitellogenic events in the female mosquito (Cheon et al., 2001).

The pattern of *Aa*VgR mRNA expression in the ovaries is similar to that of *Aa*LpRov, but *Aa*VgR mRNA is expressed at higher levels in both previtellogenic and early vitellogenic ovaries (Cheon et al., 2001; Cho and Raikhel, 2001). According to the deduced amino acid sequences, *Aa*LpRov has eight Class A cysteine-rich repeats in a ligand binding domain like the vertebrate VLDLR. Following the ligand binding domain is an EGF precursor homology domain and between the EGF precursor domain and the transmembrane domain, the receptor is serine- and threonine- rich thus containing multiple potential sites for O-linked sugar chains. Finally, the putative

cytoplasmic tail of the receptor contains a highly conserved internalization signal, NPXY. The deduced amino acid sequence of *Aa*LpRov is most similar to that of the locust lipophorin receptor (63% identity) (Dantuma et al., 1999; Cheon et al., 2001) and is only distantly related to the *Aa*VgR (18.3% identity) that has a different ligand (Sappington et al., 1996; Cheon et al., 2001).

2.5.4. *Caenorhabditis elegans* VgR

Another reported invertebrate VgR is the RME-2 of the nematode, *Caenorhabditis elegans*. It was characterized based on its mutant phenotype, expression pattern, molecular nature and sufficiency to induce yolk binding in a heterologous cell type (Grant and Hirsh, 1999). The full-length *rme-2* mRNA size of 2.9 kb encodes a protein of 925 amino acids in length with a Mr. of ~110 kDa shown by western blot analysis. RME-2 contains several repeated sequence motifs, with overall similarity to members of the LDLR superfamily. There are five, tandem Class A cysteine-rich repeats in the N-terminal ligand binding domain, each containing six cysteine residues with characteristic spacing and typical anionic SDE or DDE sequences. Following the ligand binding domain is an EGF precursor homologous domain and a transmembrane domain. Finally, within the predicted intracellular domain, there is an internalization signal of the NPXY type. Several mutants of *rme-2* were sequenced. These mutants showed partial deletion of the YWTD region, the Class B cysteine-rich repeats 3 and 4 and the predicted transmembrane and intracellular domains. Such mutated proteins are unlikely to retain any endocytotic function (Grant and Hirsh, 1999).

2.6. The LDLR-related protein (LRP)

The human LRP is a hetero-dimeric protein consisting of 515- and 85-kDa subunits. It is synthesized as a single polypeptide of 4525 amino acids and is cleaved by an endoprotease, furin, in the Golgi compartment to produce two subunits of 3924 and 601 amino acids (Herz et al., 1990; Willnow et al., 1996). The 515-kDa subunit contains 31 Class A cysteine-rich repeats that are organized in four domains (Fig. 2-3). See number 6. LRP is a multi-ligand receptor and the ligands include lipoproteins (apoE, chylomicro remnants), protease inhibitors (α2-macroglobulin) and plasminogen activators (Krieger and Herz, 1994). Studies suggest that LRP binds and internalizes proteins associated with Alzheimer's disease (AD) such as amyloid precursor protein (APP) (Jordan et al., 1998; Narita et al., 1997). AD is a neurodegenerative disease that results in impaired memory and cognition. The hallmarks of AD are amyloid plaques and neurofibrillary tangles deposited in the brain (Price et al., 1998). The major components of amyloid plaques are amyloid β-peptides (Aβ) that are proteolytically derived from the APP (Glenner and Wong, 1984). LRP ligands, such as apoE and α2-macroglobulin, have been shown to bind Aβ and to mediate its clearance and degradation through LRP (Jordan et al., 1998; Narita et al., 1997). Reduced LRP expression in older adults and in AD patients is correlated with increased soluble Aβ levels and amyloid deposition (Kang et al., 2000). Thus the loss of LRP function to clear ligands, complexed with Aβ, may contribute to the appearance of amyloid plaques in AD and aging populations.

Wnt (an amalgam of wingless and int) proteins form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during

embryogenesis. The Wnt family of secreted molecules functions in cell-fate determination and morphogenesis during development in both vertebrates and invertebrates (Wodarz and Nusse, 1998). *Drosophila* Wingless is a member of this family, and the Frizzled (Fz) family of serpentine (7 TM; GPCRs) receptors function as Wnt receptors. In *D. melanogaster*, *arrow* encodes a single-pass transmembrane protein indicating that it may be part of a receptor complex with Fz family proteins (Wehrli et al., 2000). *arrow* phenocopies the wingless phenotype and encodes a protein of 1678 amino acids exhibiting a striking sequence conservation to the mammalian LRP5 and LRP6 (Wehrli et al., 2000). In mouse embryos homozygous for an insertional mutation in the LRP6 gene, developmental defects are seen that display a striking composite of those caused by mutations in individual Wnt genes (Pinson et al., 2000). In *Xenopus* embryos, LRP6 activates Wnt-Fz signaling, and induces Wnt-responsive genes, dorsal axis duplication, and neural crest formation (Tamai et al., 2000). Biochemical studies showed that the extracellular domain of LRP6 bound Wnt-1, and associated with Fz in a Wnt-dependent manner. An LRP6 mutant, lacking the carboxyl intracellular domain, blocked signaling by Wnt or Wnt-Fz, and inhibited neural crest development (Tamai et al., 2000). These studies thus demonstrate that LRP6 functions as a co-receptor for Wnt signal transduction with Fz.

2.7. Megalin/gp330

In vertebrates, megalin is essential for development of the forebrain by taking up apoB-containing lipoproteins into the embryonic neuroepithelium (Willnow et al., 1996). *In vitro*, megalin binds many of the ligands that are also known to bind to LRP

including lipoproteins, lipoprotein lipase, and urokinase (Willnow et al., 1992). Despite their partial overlap in ligand spectrum, the spatial and temporal differences in the expression patterns for megalin and LRP suggest distinct physiological roles. Megalin is a single polypeptide of 4660 amino acids containing four clusters of ligand binding domains consisting of 7, 8, 10 and 11 Class A cysteine-rich repeats in the molecule that recognize several groups of ligands (Fig 2-3). See number 7. The intracellular domain contains two internalization signals, as observed in other members, but its overall sequence is different from that of other receptors. The intracellular domain of megalin contains Src (sarcoma)-homology binding regions, casein kinase II sites, and protein kinase phosphorylation sites, indicating that it may be involved in signal transduction (Christensen et al., 1998).

2.8. *Caenorhabditis elegans* receptor

A > 23 kb gene that encodes a large integral membrane protein with a predicted structure and function similar to those of megalin has been isolated and sequenced from the nematode *C. elegans*. The gene is called *lrp-1* (LDLR-related protein-1) (Yochem and Greenwald, 1993; Yochem et al., 1999). The 4753-amino acid predicted *C. elegans* product shares a nearly identical number and arrangement of amino acid motifs with megalin (Fig. 2-3). See number 8. The LRP-1 is essential for growth and development of the nematode. The mutations of LRP-1 confer a striking defect: an inability to shed and degrade all of the old cuticle at each of the larval molts (Yochem et al., 1999).

CHAPTER III

CDNA CLONING AND SEQUENCE ANALYSIS OF THE VITELLOGENIN RECEPTOR FROM THE IMPORTED FIRE ANT, *SOLENOPSIS INVICTA* BUREN (HYMENOPTERA: FORMICIDAE)

1. INTRODUCTION

Developing oocytes of oviparous animals accumulate large amounts of the extra-ovarian yolk protein precursor, vitellogenin (Vg), during oogenesis. Vg is synthesized by the fat body of insects or the liver of vertebrates for release into the circulatory system and transport to the ovaries. The selective internalization of Vg by the growing oocyte is achieved through receptor-mediated endocytosis (Raikhel and Dhadialla, 1992). Receptor-mediated entocytosis of Vg into the oocyte has been described for a number of insect species including: *Locusta migratoria* (Rohrkasten and Ferenz, 1986b), *Nauphoeta cinerea* (Konig and Lanzrein, 1985), *Manduca sexta* (Osir and Law, 1986), *Blattella germanica* (Konig et al., 1988), *Rhodnius prolixus* (Wang and Davey, 1992), and *Aedes aegypti* (Dhadialla and Raikhel, 1991).

The sequences of several vertebrate and invertebrate VgRs are known. For vertebrates, VgRs are described for the chicken, *Gallus gallus* (Bujo et al., 1994); the toad, *Xenopus laevis* (Okabayashi et al., 1996); the rainbow trout, *Oncorhynchus mykiss* (Davail et al., 1998) and the fish, *Oreochromis auresu* (Li et al., 2003). For invertebrates, they are described for the fruit fly, *Drosophila melanogaster* (Schonbaum et al., 1995); the mosquito, *A. aegypti* (Sappington et al., 1996); the mosquito,

Anopheles gambiae (accession number XP_310672); the American cockroach, *Periplaneta* a*mericana* (accession number BAC 02725) and the nematode, *Caenorhabditis elegans* (Grant and Hirsh, 1999). All of these VgRs are members of the low-density lipoprotein receptor (LDLR) superfamily. An aspect of this superfamily is that the physiological roles of its members are diverse as reflected by a wide range of ligands (Schneider et al., 1999; Nykjaer and Willnow 2002; Strickland et al., 2002), even though they have many structural elements in common. These common structural elements are: i) the ligand-binding domain comprised of Class A cysteine-rich repeats; ii) the epidermal growth factor (EGF) precursor homology domain containing Class B cysteine-rich repeats and YWXD repeats; iii) an O-linked carbohydrate domain; iv) a transmembrane domain; and v) a cytoplasmic tail (Goldstein et al., 1985; Schneider, 1996).

The size and structure of insect VgRs are different from those of vertebrate VgRs. Insect VgRs (190-214 kDa) are about twice the size of the vertebrate VgRs (95-115 kDa) (Dhadialla et al., 1992; Bujo et al., 1994; Okabayashi et al., 1996; Sappington et al., 1996; Davail et al., 1998). The *A. aegypti* VgR (*Aa*VgR) and *D. melanogaster* yolk protein receptor (*Dm*YPR) each contains two ligand-binding domains comprised of five and eight Class A cysteine-rich repeats respectively, unlike vertebrate VgRs which have a single ligand binding domain comprised of eight Class A cysteine-rich repeats (Bujo et al., 1994; Okabayashi et al., 1996; Davail et al., 1998). In addition, the two insect VgRs contain two EGF precursor homology domains rather than only one as presented in the vertebrate VgRs.

In this chapter the *S. invicta* VgR (*Si*VgR) cDNA is cloned and sequenced. The entire coding region sequence is analyzed and subjected to phylogenetic analyses.

2. MATERIALS AND METHODS

2.1. Insects

Polygyne colonies of *S. invicta* were obtained from the Fire Ant Research Laboratory in the Department of Entomology (Texas A&M University, College Station, TX). All colonies were collected in Brazos County, Texas. The laboratory colonies of *S. invicta* were housed in plastic trays (27 x 40 x 9 cm) containing one nest in a 14 cm diameter Petri dish half-filled with damp Castone® (Dentsply International Inc., York, PA). The colonies were maintained at 27 ± 2°C in a 12L:12D photoperiod and fed daily with 20% honey-water, cockroach carcasses (*Blaberus discoidalis*) and an artificial diet (Kuriachan & Vinson, 2000). Water was given *ad lib*.

2.2. Cloning the fire ant VgR cDNA by RT-PCR and RACE

Ovaries were collected from queens 10 days after insemination, and total RNA was extracted using the guanidine-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987; Puissant and Houdebine, 1990). The ovarian total RNA (1 μg) provided templates for reverse transcription (RT) reaction using oligo-dT_{16} primers with a GeneAmp® RNA PCR Core kit (Perkin Elmer, Vellesley, MA). To obtain cDNA fragments containing the coding region of VgR, polymerase chain reaction

(PCR) was applied following RT reaction using an oligo-dT_{16} and one degenerate oligonucleotide, which was designed based on the consensus sequences of the *A. aegypti* and *D. melanogaster* VgRs (Schonbaum et al., 1995; Sappington et al., 1996). PCR parameters were 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min; 72°C for 10 min. The PCR product (2.3kb) was purified using QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA), and cloned into pGEM®-T Easy Vector (Promega, Madison, WI). The cDNA fragment was sequenced using ABI PRISM® Big Dye Terminator Cycle sequencing Core kit with AmpliTaq® DNA Polymerase (Perkin Elmer) and the sequences were obtained by the Gene Technology Laboratory (Texas A&M University, College Station, TX). Big Dye sequence results were compared with other protein sequences available through the database of the National Center for Biotechnology Information (NCBI) BLAST e-mail server (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/BLAST).

The 5' end of the *Si*VgR cDNA fragment was produced using the 5' RACE system (Gibco BRL®, Invitrogen, Carlsbad, CA) and Gene Racer™ Kit (Invitrogen™, Carlsbad, CA). *Si*VgR-specific primers were designed based on the 5'-end sequence of the 2.3 kb fragment. Manufacturer's instructions were followed. The resulting bands were purified, cloned, and sequenced as described above.

The 3' end of the *Si*VgR cDNA fragment was produced using the Gene Racer™ Kit (Invitrogen™, Carlsbad, CA). mRNA was purified from ovaries using Dynabeads® mRNA DIRECT Kit (DYNAL®, Oslo, Norway). *Si*VgR-specific primers were designed based on the 3'-end sequence of the 2.3 kb fragment. PCR parameters were

94°C for 30 s; 35 cycles of 94°C for 30 s, and 68°C for 2 min 30 s; 68°C for 10 min. Manufacturer's instructions were followed. The resulting bands were purified, cloned and sequenced as described above. All cDNA fragments were sequenced at least twice.

2.3. Phylogenetic analysis and amino acid sequence comparison

Translation of the nucleotide sequence was accomplished by the Translator through the MBS – Molecular Biology Shortcuts e-mail server (http://www.justbio.com/translator/index.php). The SMART (simple modular architecture research tool) (Schultz et al., 1998; Letunic et al., 2002) (http://smart.embl-heidelberg.de) was used for identification of modular domains that were adjusted by eye, when necessary. Percent identity and similarity for subdomains were determined by the EMBOSS Pairwise Alignment Algorithms of EBI (European Bioinformatics Institute) (http://www.ebi.ac.uk/emboss/align).

The amino acid sequences of ten VgRs, four VLDLRs and two LDLR-related proteins (LRP) were multiple-aligned and the phylogram among the sixteen LDLR superfamily members listed above were performed using the Clustal W computer program (Thompson et al., 1994) (http://www.ebi.ac.uk/clustalw).

The accession numbers for the receptors used for multiple alignment and phylogenetic analyses are as follows: chicken VgR P98165, frog VgR JC4858, rainbow trout VgR CAD10640, fish VgR AAO27569, mosquito *Aedes aegypti* VgR T30347, mosquito *Anopheles gambiae* VgR XP_310672, fruit fly VgR P98163, American cockroach BAC02725, fire ant VgR AY262832, nematode VgR NP500815, human

VLDLR P98155, rabbit VLDLR P35953, mosquito VLDLR AAK72954, locust VLDLR CAA03855, human LRP Q07954, and nematode LRP, CAA98124.

3. RESULTS AND DISCUSSION

3.1. Cloning and sequence analysis of the cDNA for the *S. invicta* VgR

S. invicta VgR cDNA was cloned using RT-PCR, 5' and 3' RACE as described in Materials and Methods. Comparisons were made with other protein sequences available through the National Center for Biotechnology Information BLAST e-mail server (Altschul et al., 1990). The deduced amino acid sequence of the first PCR fragment obtained (2.3 kb) indicated that it was highly similar to mosquito and fruit fly VgRs. The 2.3 kb fragment was used to design specific primers to extend the 5' and 3' ends of the *Si*VgR-cDNA. cDNA fragments encompassing the entire coding region were cloned and sequenced (Fig. 3-1).

The 5764 bp cDNA sequence revealed an open reading frame of 5349 bp encoding a protein of 1782 amino acid residues. There were two possible start codons at the 5' end at positions 107 and 113. Based on the Kozak sequence, the start codon at position 113 conformed most closely to the initiation site consensus sequence (Kozak, 1984). A stretch of 16 predominantly hydrophobic residues defined a signal peptide downstream of the methionine start codon (Fig. 3-1). The open reading frame predicted a protein with molecular mass of 201.3 kDa and an isoelectric point of 5.5. There are several possible sites for co- and posttranslational modification of the *Si*VgR, including 13 N-linked glycosylation sites. The *Si*VgR colud be heavily phosphorylated on Ser with

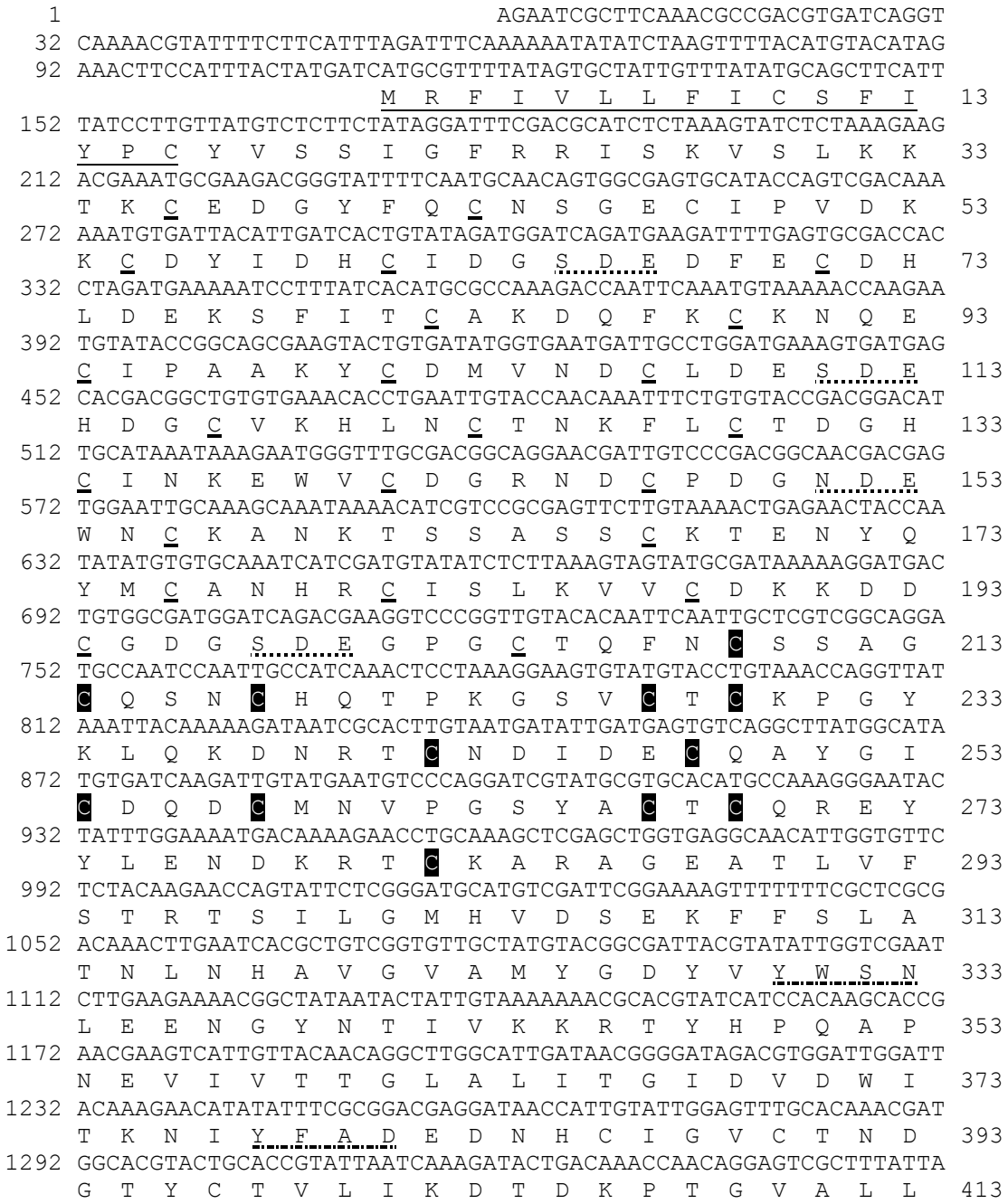

Fig. 3-1. Nucleotide sequence and deduced amino acid sequence of the vitellogenin receptor cDNA of *S. invicta*. The putative signal sequence at the N-terminus and a presumed transmembrane sequence near the C-terminus are underlined and double underlined, respectively. Class A cysteine residues are indicated as C and Class B cysteine residues as C. The SDE sequences are present as SDE. The YWXD or potentially related sequences present in YWXD. The internalization signal, NPSY is in boldface type.

```
1352 CCAACCCAAGGAAAAATGTATTGGAGCGATTGGGGTACTTTTCCACATATAGCAGTGGCC
     P  T  Q  G  K  M  Y  W  S  D  W  G  T  F  P  H  I  A  V  A   433
1412 GGAATGGATGGTAAAAACGTCCGTATATTCGTCAATGTAAAGTTAGAATGGCCGAAAAGT
     G  M  D  G  K  N  V  R  I  F  V  N  V  K  L  E  W  P  K  S   453
1472 GTCACTATTGATTATCCGAACGAGAGATTATATTGGGTGGATGCCAAGTCAAAGATGATC
     V  T  I  D  Y  P  N  E  R  L  Y  W  V  D  A  K  S  K  M  I   473
1532 GAATCTGTACGCTTAGACGGCACTGATCGAAGAATAGTGTTACATGATATAATACAAGAG
     E  S  V  R  L  D  G  T  D  R  R  I  V  L  H  D  I  I  Q  E   493
1592 CCATTCTCTATGACGGTCTTTCAAAATAAATTATACTGGAGCGATTGGGAATCCAATGGG
     P  F  S  M  T  V  F  Q  N  K  L  Y  W  S  D  W  E  S  N  G   513
1652 ATACAGACCTGCAATAAATTTACTGGAAAGGATTGGAAGATCTTAATTCGTAATCATAAT
     I  Q  T  C  N  K  F  T  G  K  D  W  K  I  L  I  R  N  H  N   533
1712 AAACCTTATAGCGTGCACATGGATCATTCAGCGATAAAACCTAATATTGACAATCCATGT
     K  P  Y  S  V  H  M  D  H  S  A  I  K  P  N  I  D  N  P  C   553
1772 TACTCTAATCCGTGTTCTCAATTATGCATGTTGAATCAGAATAAAGGTTACACGTGCGGC
     Y  S  N  P  C  S  Q  L  C  M  L  N  Q  N  K  G  Y  T  C  G   573
1832 TGCACTTTGGATAAGAAATTAAATGCCGACAAACATACTTGTCAAGATGTGAAGAAGAAT
     C  T  L  D  K  K  L  N  A  D  K  H  T  C  Q  D  V  K  K  N   593
1892 CAGCATTTATTAATTATTCAAGGAAGAAAATTCATTAACTACTATCACGAATTCTTAGGA
     Q  H  L  L  I  I  Q  G  R  K  F  I  N  Y  Y  H  E  F  L  G   613
1952 AAGCCAAAAGTAATGACATTGTCACTGCAACACATGTCACAACAATCATATAACAATCTA
     K  P  K  V  M  T  L  S  L  Q  H  M  S  Q  Q  S  Y  N  N  L   633
2012 GTGAATATAATATCTGATCCGCTCTCAGGTCAGATAATTATTTGCCATCTGCAATTATCA
     V  N  I  I  S  D  P  L  S  G  Q  I  I  I  C  H  L  Q  L  S   653
2072 ACGCCTTTCTTAACGTCAACGACAGACATCTTACGCTATGATCCAGTCCACCACAGTTCG
     T  P  F  L  T  S  T  T  D  I  L  R  Y  D  P  V  H  H  S  S   673
2132 GAAAAAATAGTGACGATCAATAAGATATTTTTCGAATTAGCATTTGATTATATTGGCAAT
     E  K  I  V  T  I  N  K  I  F  F  E  L  A  F  D  Y  I  G  N   693
2192 AACTTGTACACAACAAATACTGTAAACCAATCTATCGAGGTAATCAACTTAAACACCAAG
     N  L  Y  T  T  N  T  V  N  Q  S  I  E  V  I  N  L  N  T  K   713
2252 GCAATGACGGCCTTTTATTTTAAGGATGAGGTTCCTAAATATATTGCACTAGCTCCTGAA
     A  M  T  A  F  Y  F  K  D  E  V  P  K  Y  I  A  L  A  P  E   733
2312 GAAAGCAAGATGTTCGTAGCCTTTCAAAAATCAATGCATTCGATCAGTGGTTTGACTTTA
     E  S  K  M  F  V  A  F  Q  K  S  M  H  S  I  S  G  L  T  L   753
2372 TATGAGATGCAAATGAACGGACTCGGTAAAAGAAAGCTAATTAGGGAAGGATTAATTGGT
     Y  E  M  Q  M  N  G  L  G  K  R  K  L  I  R  E  G  L  I  G   773
2432 CCACAATTACCAATGTACTATGACAGAGATAGTAAAACACTTTTTGTGAGTGATTTGCTC
     P  Q  L  P  M  Y  Y  D  R  D  S  K  T  L  F  V  S  D  L  L   793
2492 CCAGGTTACATTTACTCGCATTCGGCCCAAGACACGCGTATACTTCGTTCTGGACTAAAG
     P  G  Y  I  Y  S  H  S  A  Q  D  T  R  I  L  R  S  G  L  K   813
2552 AGCCCCCACAGTCTTACTGTCGCCGGCGACAATCTTTTCTGGATCGAATCACAGAATAAA
     S  P  H  S  L  T  V  A  G  D  N  L  F  W  I  E  S  Q  N  K   833
2612 TTATACTCGACAAATTTTAGAACAGCCTCTGTAAAACAGAAGACTGTTGAGTTTGATCTC
     L  Y  S  T  N  F  R  T  A  S  V  K  Q  K  T  V  E  F  D  L   853
2672 TCTAAATTAAACGATAATATGACATCTTTACCTGGTCATTTGACACCTTATTCACGCGAT
     S  K  L  N  D  N  M  T  S  L  P  G  H  L  T  P  Y  S  R  D   873
2732 GCACAGTATGTGGTAACTCTGCGCAAAGATGATATACCAAAGCACGATTGTCAGAAAAAT
     A  Q  Y  V  V  T  L  R  K  D  D  I  P  K  H  D  C  Q  K  N   893
2792 AATGGAAATTGTTCACACGTGTGTTTGCCTTCTCTTATTACTTCATTTATCTGTGCTTGT
     N  G  N  C  S  H  V  C  L  P  S  L  I  T  S  F  I  C  A  C   913
2852 CCTCCTGGAATGGAACTATCAAATGATAATCGTACATGCATTTCGCATCATGAATGTTCT
     P  P  G  M  E  L  S  N  D  N  R  T  C  I  S  H  H  E  C  S   933
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Fig. 3-1. Continued.

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2912 AAGAACGAATATAAATGTAGCGAGCACAACATCTGCATACAAAGAAACCAATTGTGTGAT
     K  N  E  Y  K  C  S  E  H  N  I  C  I  Q  R  N  Q  L  C  D   953
2972 GGCATCGAAAATTGCCCGAACGGTGAAGACGAAACGAGTGAATGCAGAATAAAAGGAAGA
     G  I  E  N  C  P  N  G  E  D  E  T  S  E  C  R  I  K  G  R   973
3032 TGCAAGGAAAATCAGTTCATGTGTAAAAATGGCGACTGCATCAGGTTAAAAGATCGTTGT
     C  K  E  N  Q  F  M  C  K  N  G  D  C  I  R  L  K  D  R  C   993
3092 AACTCGCGTTACGATTGTACCGATCAATCGGACGAGCAGAACTGCGAGAAACCGAAATGC
     N  S  R  Y  D  C  T  D  Q  S  D  E  Q  N  C  E  K  P  K  C  1013
3152 AAAAGTGATGAATTTCAATGTAAATTTACTGAGACTTGCATACCGAAGACCAAAATGTGC
     K  S  D  E  F  Q  C  K  F  T  E  T  C  I  P  K  T  K  M  C  1033
3212 GACAGCAATCCTGATTGTGACGATTTAAGTGATGAGGAAGATTGCAGGAAAGTAGAGTGC
     D  S  N  P  D  C  D  D  L  S  D  E  E  D  C  R  K  V  E  C  1053
3272 ACTTCAAACGAATTCAAGTGCAACAATGGTAAATGCATTCCAAACACATTTGTATGCGAC
     T  S  N  E  F  K  C  N  N  G  K  C  I  P  N  T  F  V  C  D  1073
3332 AACGATAACGATTGTGAGGATGGAGAGGATGAAGCAGCCGAGAAATGTTACTCAAAGATA
     N  D  N  D  C  E  D  G  E  D  E  A  A  E  K  C  Y  S  K  I  1093
3392 GCATGTAAAATGCCTAAGATGTTTAAGTGCCCAAATGGTGATTGTATAAGTGACTCGTTA
     A  C  K  M  P  K  M  F  K  C  P  N  G  D  C  I  S  D  S  L  1113
3452 TTATGCAATGGCATAAACGATTGCAACGATGGTAGCGATGAGGTGCACTGCTTAAGTAAT
     L  C  N  G  I  N  D  C  N  D  G  S  D  E  V  H  C  L  S  N  1133
3512 GTAACAACTCATTTGGTTAATTGCAGTTTAAATGAATATCGGTGTCTCGGCACTGATATA
     V  T  T  H  L  V  N  C  S  L  N  E  Y  R  C  L  G  T  D  I  1153
3572 TGTCTTCCAAAAAATGTAAGATGCGACGGCAAAAATGATTGTCCGCAATCAGATGACGAG
     C  L  P  K  N  V  R  C  D  G  K  N  D  C  P  Q  S  D  D  E  1173
3632 CAAAATTGCACGTATTGTTTCGAGAATGAATTTGCCTGCGATAATAAAAGGTGCATTCCG
     Q  N  C  T  Y  C  F  E  N  E  F  A  C  D  N  K  R  C  I  P  1193
3692 GAGCTTTGGGTATGCGACAAGGCCAATGATTGTGGCGACAATTCGGATGAGAAGAATTGC
     E  L  W  V  C  D  K  A  N  D  C  G  D  N  S  D  E  K  N  C  1213
3752 GATGGCAGTAAAAGGAACTTCATCGAATCTAACGAATGTGATGAATTTAAATGTTCTGTC
     D  G  S  K  R  N  F  I  E  S  N  E  C  D  E  F  K  C  S  V  1233
3812 GGCACTTGTTTGCCGTACAGCAAAGTATGCGACGGTAATCGAGACTGTCCAGATGGCAGC
     G  T  C  L  P  Y  S  K  V  C  D  G  N  R  D  C  P  D  G  S  1253
3872 GATGAAACCGGAAAATGTCAAACGGCCTGTACGGTGAACAATTTCTGTAAAGGTATGTGT
     D  E  T  G  K  C  Q  T  A  C  T  V  N  N  F  C  K  G  M  C  1273
3932 TACAAGACACCAGCAGGCGCAGTTTGCGGATGTCAATCAGGATATCGCTTAGCTGTCGAC
     Y  K  T  P  A  G  A  V  C  G  C  Q  S  G  Y  R  L  A  V  D  1293
3992 ATGATTTCCTGCGAGGACATAAATGAATGCGAATTAGATATTTGTTCGCAAATGTGTCGG
     M  I  S  C  E  D  I  N  E  C  E  L  D  I  C  S  Q  M  C  R  1313
4052 AACACTATAGGCTCTTACGAGTGTTTCTGTAAAGACGAATTTATTATTCGCAACGATAAA
     N  T  I  G  S  Y  E  C  F  C  K  D  E  F  I  I  R  N  D  K  1333
4112 ACCTCTTGCAAAGCTGTCGGGCCGGCAATGGAATTTATCACTGTTACTGATAATGATATT
     T  S  C  K  A  V  G  P  A  M  E  F  I  T  V  T  D  N  D  I  1353
4172 AGGAAGATGACGCACAATTTACATTCAACTACTCAATTACTCTTTCCGTTAATGGGCGTA
     R  K  M  T  H  N  L  H  S  T  T  Q  L  L  F  P  L  M  G  V  1373
4232 AGAGTAAGTGGTCTCGACGTGAACGCAGTCAGTGATTCTGTTTATTGGAGTAATGATGAA
     R  V  S  G  L  D  V  N  A  V  S  D  S  V  Y  W  S  N  D  E  1393
4292 TTCGGCACTATTAAAAAACTCAACATTAGAACGAATGAAATAGTGACAGTTAAAATTGTC
     F  G  T  I  K  K  L  N  I  R  T  N  E  I  V  T  V  K  I  V  1413
4352 GAGCATCCACAGGCACTCGCGGTTGATTGGATTACTGGTAACGTCTACGTCAATGATAAT
     E  H  P  Q  A  L  A  V  D  W  I  T  G  N  V  Y  V  N  D  N  1433
4412 AGCCATCTGAATACGATTAAGGTATGCAATTTGGAGAAAGGAAAATGTGCGACGCTTGTT
     S  H  L  N  T  I  K  V  C  N  L  E  K  G  K  C  A  T  L  V  1453
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Fig. 3-1. Continued.

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4472 AAAATACAGGATAAAATGAAAGTGGCGTCCGTCATAGTTGATTCGATTAACAGATGGTTA
     K  I  Q  D  K  M  K  V  A  S  V  I  V  D  S  I  N  R  W  L   1473
4532 TTCTGGGCTGAAATATCTTTGGAGGCAGATCATCCAACCAGCAAAATATGTCGAACAGAT
     F  W  A  E  I  S  L  E  A  D  H  P  T  S  K  I  C  R  T  D   1493
4592 ATGACGGGTGCTGACATGAAGATTATTGCTTCCGATCTGGGTTTTGTGAGAGGAATGACA
     M  T  G  A  D  M  K  I  I  A  S  D  L  G  F  V  R  G  M  T   1513
4652 ATCGATCATGTAAAATCTAAATTATATTGGTCGGACGATTTCTATAAAACCGTCGAGTCA
     I  D  H  V  K  S  K  L  Y  W  S  D  D  F  Y  K  T  V  E  S   1533
4712 TCTAATTTTGATGGAAGTCAACGCAAAGTGGTCTTAACTTTAAATATGAATCACGCGTTA
     S  N  F  D  G  S  Q  R  K  V  V  L  T  L  N  M  N  H  A  L   1553
4772 AGCATTAGTATTTTTGAACAGTCTCTTTATTTTTTGAGTTCGGACAATCTACTAAGCAGC
     S  I  S  I  F  E  Q  S  L  Y  F  L  S  S  D  N  L  L  S  S   1573
4832 TGTAAGATGTACGGTAAAAGATCGTGTGAACACGTAAACATAGGCGCAAATAATGTTTTC
     C  K  M  Y  G  K  R  S  C  E  H  V  N  I  G  A  N  N  V  F   1593
4892 AGGCTCTTCTCCATTCTTCATATTTCCAGACAGGTACCTTTCGCTAATCCTTGCGATGCA
     R  L  F  S  I  L  H  I  S  R  Q  V  P  F  A  N  P  C  D  A   1613
4952 GAGTATTGCGATTATATGTGTGTTTTAAAGAAGGAGAACGCAACGTGCATTTGTTCAGAT
     E  Y  C  D  Y  M  C  V  L  K  K  E  N  A  T  C  I  C  S  D   1633
5012 GGTGAATCAATAGAATCCAATAGCACGTGCAACATAAAAAATGACCTCAAATTTGTTGAA
     G  E  S  I  E  S  N  S  T  C  N  I  K  N  D  L  K  F  V  E   1653
5072 AGCATTAATTTCTCGAGAAATACACGGAACATAAGCGGCATTTATAGCATAACCATAATC
     S  I  N  F  S  R  N  T  R  N  I  S  G  I  Y  S  I  T  I  I   1673
5132 GTATTATTGGTTAGTGTTCTGTTATTATGCGTTTACTATTACTATCAGAAAAACAAATTA
     V  L  L  V  S  V  L  L  L  C  V  Y  Y  Y  Y  Q  K  N  K  L   1693
5192 AAGTCGAAGCCAGCGAGCAATCTTAGTTGCAGCAGCATTCACTTCCAAAATCCGTCGTAC
     K  S  K  P  A  S  N  L  S  C  S  S  I  H  F  Q  N  P  S  Y   1713
5252 GATCGAAGCGATGAAATTGAAGTAATGCTTGACTCCATGGCTTCTTCTGAATTATCTCCC
     D  R  S  D  E  I  E  V  M  L  D  S  M  A  S  S  E  L  S  P   1733
5312 GGACAGCACGAATATATTAATCCTATTAACAATAAAGGTATGAAGGCTGCNGAAAATAAT
     G  Q  H  E  Y  I  N  P  I  N  N  K  G  M  K  A  A  E  N  N   1753
5372 GCAAAGAAATCAAATCAATGCTCGGAGGGAAAGAATATTGAAGAGGAAAAACAGGATGCG
     A  K  K  S  N  Q  C  S  E  G  K  N  I  E  E  E  K  Q  D  A   1773
5432 TTAATTTATTTTGTACATAATTCAAAATAATTCAATTACAGTCGAAGAAATATCACAAAA
     L  I  Y  F  V  H  N  S  K  *                                  1782
5492 ATTAATATTCAAATTGAATTTAAAATTAGTCAAATTTAAATTTAAAATTAGTCAACAAAA
5552 CCTTTTATGTGACGTTAGAAAATTTCTTACTATTTTGTATCATAAATTTCAAGACGTTGT
5612 TGGTGAATGTGTGCCTATAAATGCAATATATACATAAATATTGATATATGTAACTCAATA
5672 ATTTAGACATATTTGATCACTACGAAATCAAATGGGTTACAAAATGAACTATGATCTATG
5732 TCAAATATTTCCTATGCACGATCACTTATCTTT
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Fig. 3-1. Continued.

potential 54 sites, and there are 15 Thr and 24 Tyr potential phosphorylation sites. However, there are no predicted Tyr sulfation sites.

Analyses of the deduced amino acid sequence for *Si*VgR indicated that it was a member of the LDLR superfamily. Like other members of the LDLR superfamily, the *Si*VgR was characterized by a highly conserved arrangement of modular elements (Fig. 3-2, 3-4). *Si*VgR exhibited two ligand-binding domains with four Class A cysteine-rich repeats in the first domain and eight repeats in the second domain. Each repeat contained six cysteine residues and a conserved acidic residue region, SDE, required for ligand binding. Each ligand-binding domain was followed by EGF precursor homology domains (Fig. 3-2). A hydrophobic region representing a transmembrane domain was found at amino acid residues 1668-1690. In the cytoplasmic region, there was a signal sequence, NPSY, for receptor internalization (Goldstein et al., 1985; Chen et al., 1990) (Fig. 3-1).

The deduced amino acid sequence of *Si*VgR was aligned with the other two insect VgRs, *Aa*VgR and *Dm*YPR (Fig. 3-2, 3-3). The deduced amino acid sequence of *Si*VgR showed 35% identity and 52% similarity to *Aa*VgR; 31% identity and 48% similarity to *Dm*YPR. Percentages of identity and similarity were also assessed between different modular domains (Fig. 3-2).

The *Si*VgR differed structurally from the *Aa*VgR and *Dm*YPR in the first ligand binding domain and the cytoplasmic tail. *Si*VgR contains four Class A cysteine-rich repeats in the first ligand-binding domain rather than the five repeats that are found in *Aa*VgR and *Dm*YPR (Fig. 3-2). In order to confirm that only four Class A cystine-rich repeats occur in the *Si*VgR, the 5' end of *Si*VgR cDNA was performed twice by 5'

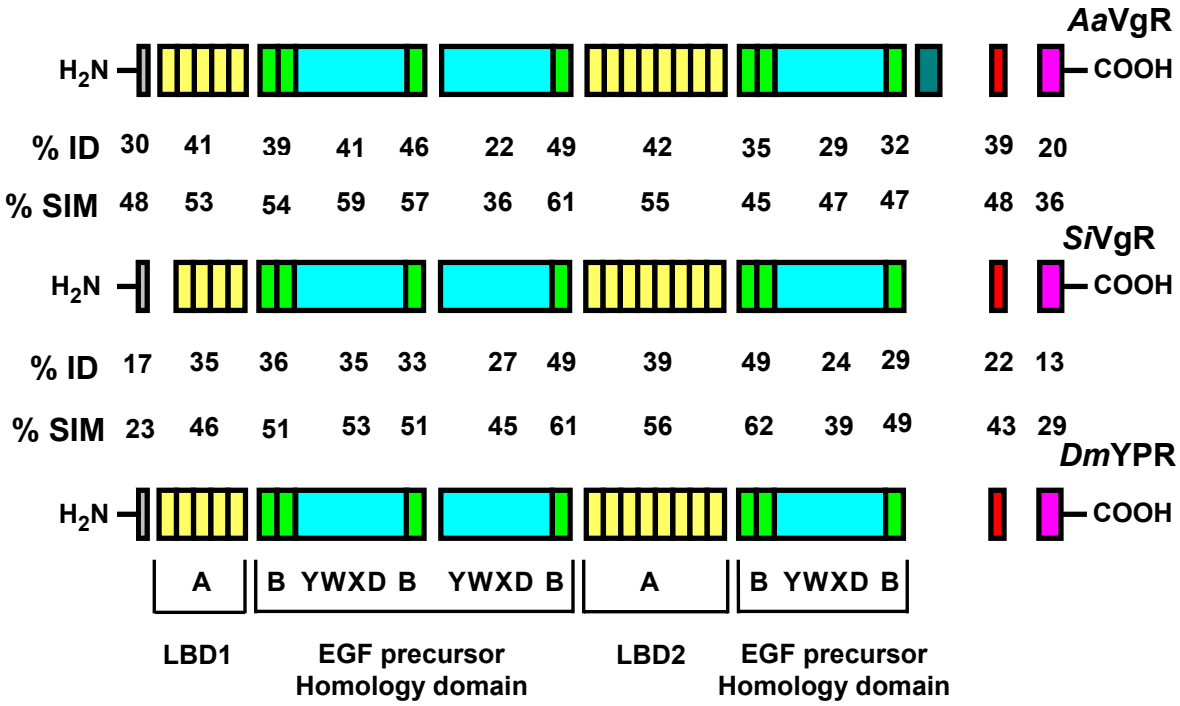


Fig. 3-2. The arrangement of modular domains for insect VgRs and the schematic alignment of *Si*VgR modular domains with those of *Aa*VgR and *Dm*YPR. Pairwise alignment was performed with EMBOSS Pairwise Alignment Algorithms of EBI. signal peptide (SP); Class A cysteine-rich repeat (A); Class B cysteine-rich repeat (B); YWXD repeat; O-linked sugar domain (*Aa*VgR only); transmembrane domain; cytoplasmic tail. LBD = ligand binding domain; the epidermal growth factor (EGF) precursor homology domain contains Class B cysteine-rich repeats and YWXD repeats; %ID = percent identity; %SIM = percent similarity.

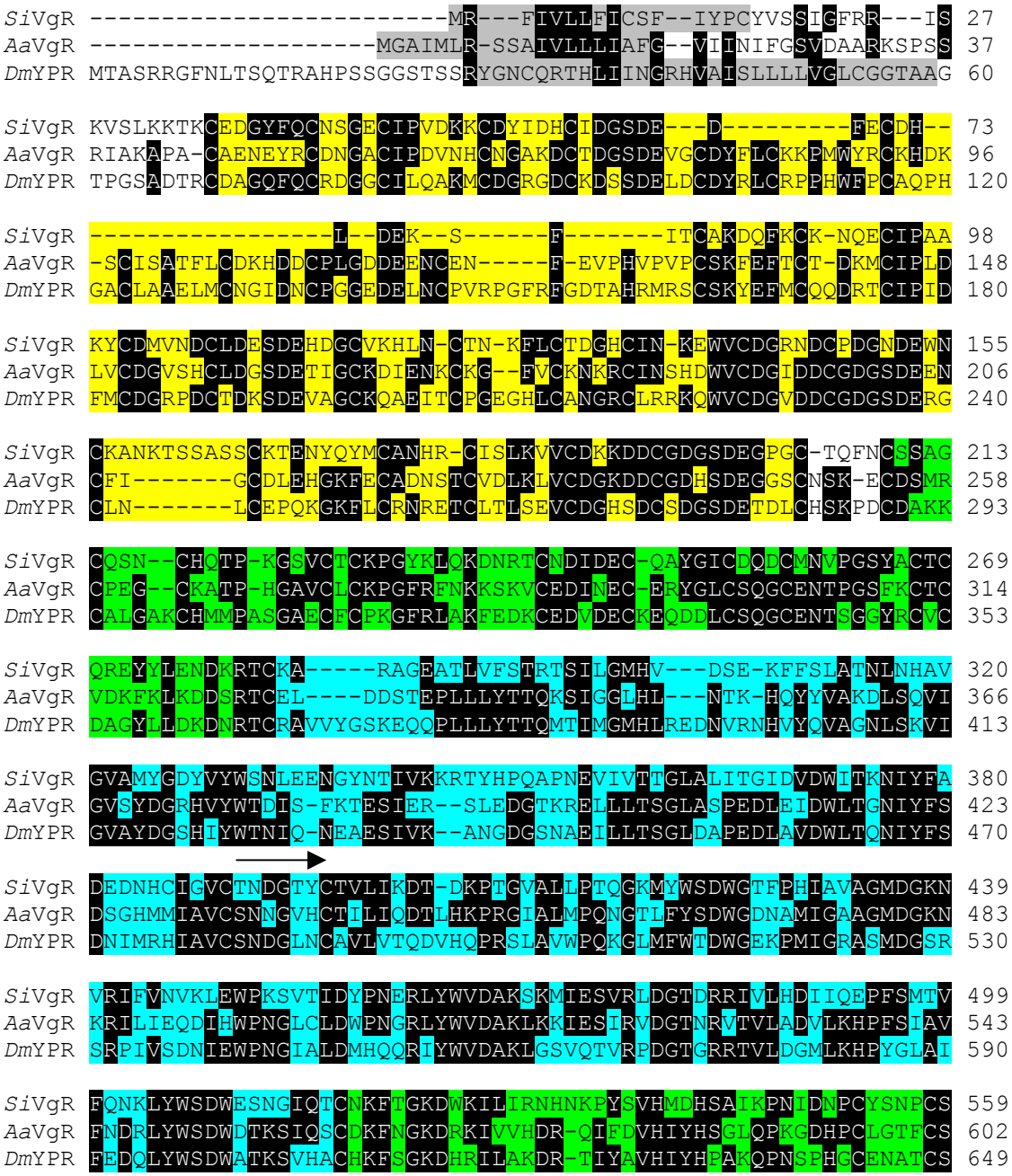


Fig. 3-3. Alignment of deduced amino acid sequence of *Si*VgR, *Aa*VgR and *Dm*YPR. Black boxes denote identical amino acids. Gray boxes indicate the signal peptide sequence. Yellow boxes indicate the ligand binding domains. Green boxes indicate the class B cysteine-rich repeats. Blue boxes indicate the YWXD repeats. The teal box indicates the O-linked sugar domain in *Aa*VgR only. Red boxes indicate the transmembrane region. Pink boxes indicate the cytoplasmic tail. The color of boxes is consistent with that in Fig. 3-2. The two over-arrows indicate forward and reverse primers using for semi-QRT-PCR in Chapter V.

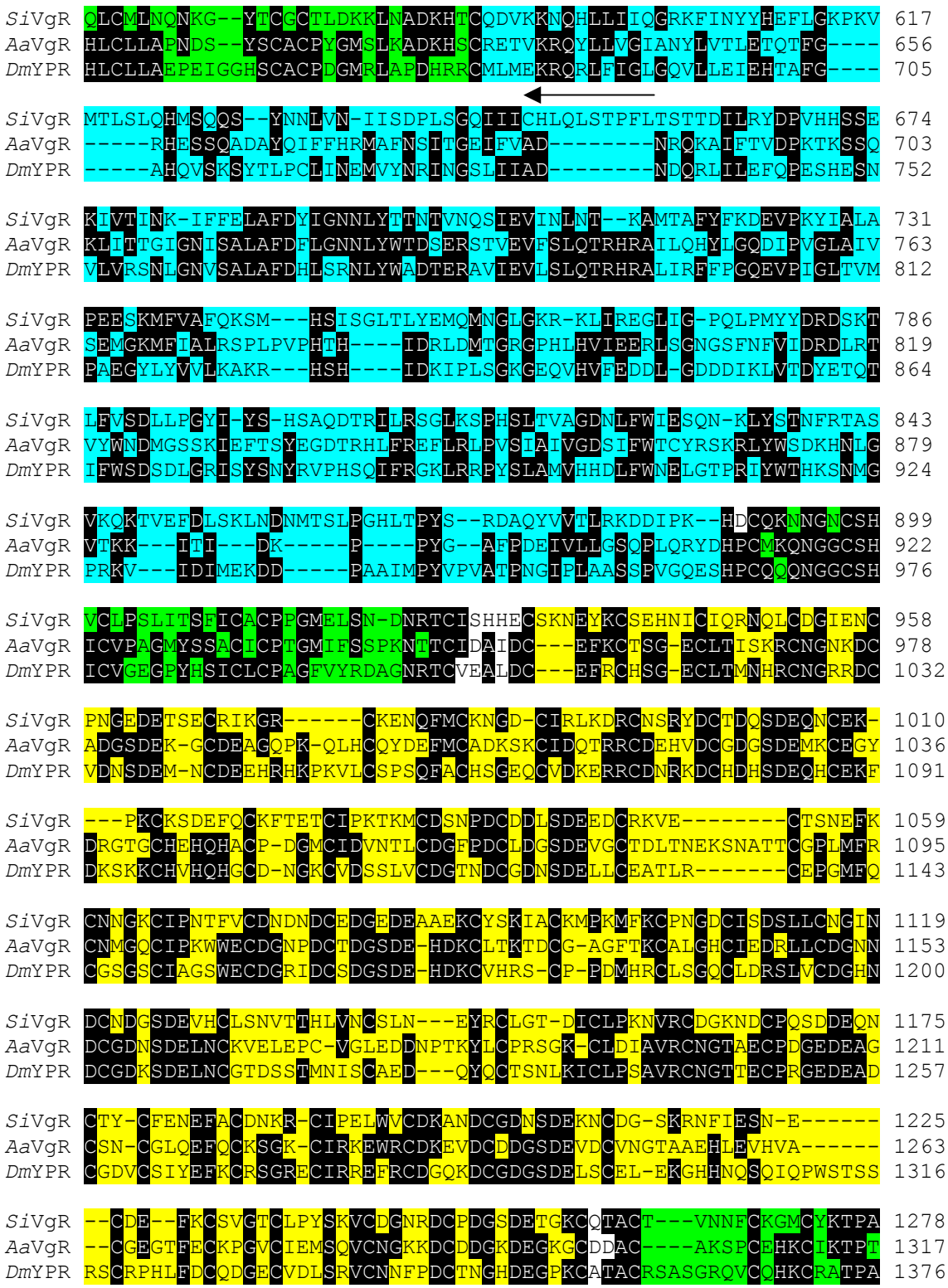


Fig. 3-3. Continued.

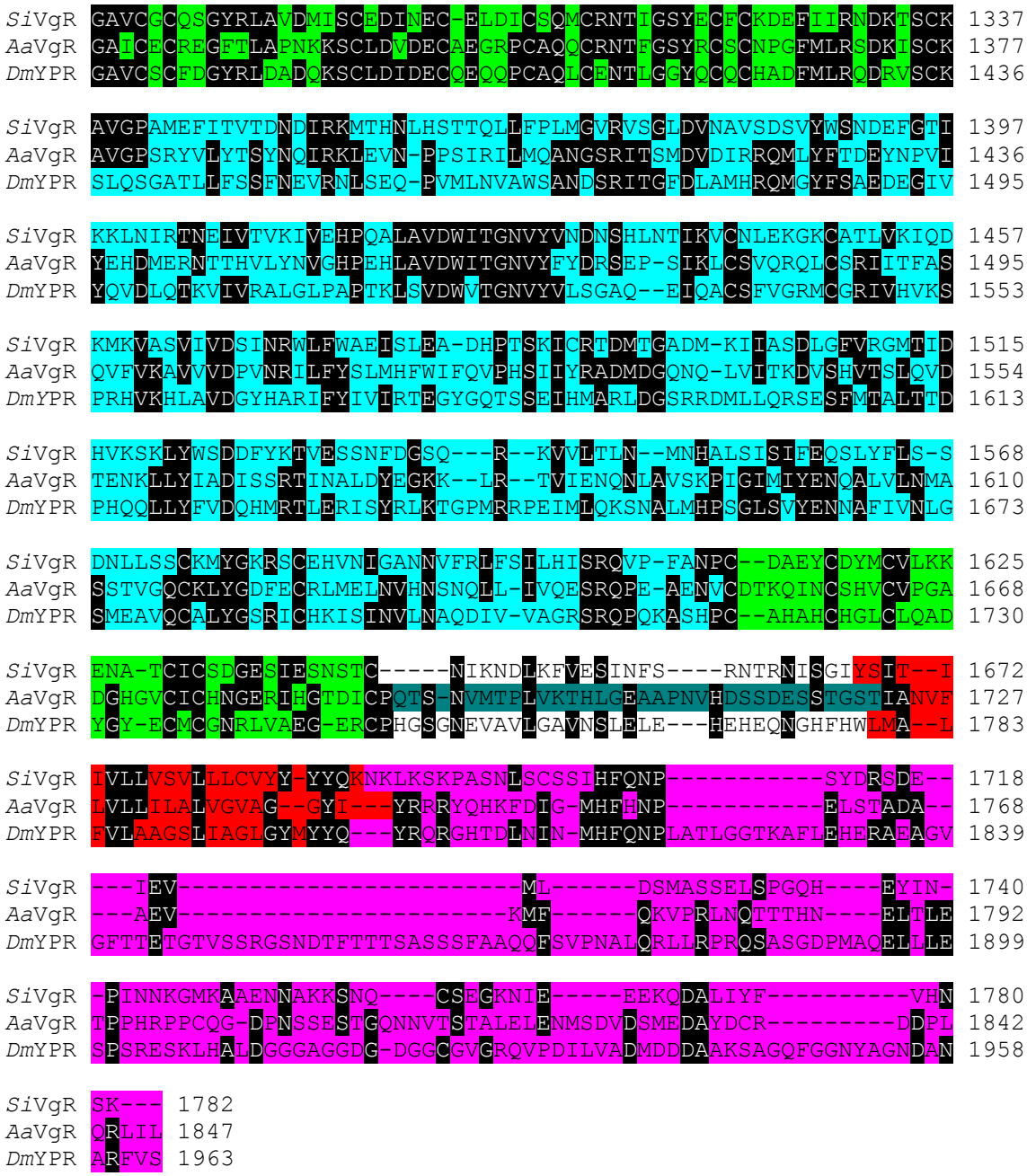


Fig. 3-3. Continued.

RACE and that produced several clones containing a >420 base-pair region of overlap between 5' RACE clones and the 2.3 kb fragment. All clones showed the same sequence. Therefore, it is likely that *Si*VgR contains only four Class A cysteine-rich repeats in the first ligand-binding domain instead of the five reported for the mosquito and fruit fly receptors. The *Si*VgR contained the typical LDLR internalization signal, NPSY, in the cytoplasmic tail, but both dipteran VgRs contain di-leucine or leucine-isoleucine motifs which are also identified as internalization signals in some receptors (Letourneur and Klausner, 1992; Dittrich et al., 1994; Sappington et al., 1996) (Fig. 3-3).

*Si*VgR and *Dm*YPR do not contain an O-linked sugar domain between the last Class B cysteine-rich repeat and the transmembrane, the consensus position for such regions in LDLR superfamily members, but *Aa*VgR does. Sakai *et al*. (1994) reported two splicing forms of the human VLDLR, one of which lacked the O-linked sugar domain. The splicing variants happen in other vertebrate VgR also. The chicken VgR gene produces an oocyte-specific VgR without an O-linked sugar domain or a soma-specific variant containing a 30-residue O-linked region (Bujo et al., 1995b). Alternative splicing of the *Xenopus* VgR gene produces an ovary-specific VgR with an O-linked sugar domain or a brain variant that is 40 bp shorter without an O-linked sugar domain (Okabayashi, et al., 1996). The rainbow trout VgR contains an O-linked sugar domain that is expressed in both the ovary and somatic tissues, whereas a VgR without an O-linked sugar domain is ovary-specific (Prat, et al., 1998). The function of the O-linked sugar domain is not known and is not universal among LDLRs (Bujo et al., 1995b; Sappington and Raikhel, 1998).

The size and structure of the *Si*VgR place it in between the LDLR/VLDLR groups with seven or eight Class A cyteine-rich repeats (such as chicken VgR), and the LRPs with 31 to 36 repeats, respectively (Fig. 2-3, 3-4). Thus, the insect VgRs form the intermediate size group in the LDLR superfamily.

3.1. VgR phylogenetic analysis

The *Si*VgR amino acid sequence was multiple-aligned with other fifteen LDLR superfamily members and the phylogram of sixteen LDLR supterfamily members was generated using the Clustal W computer program (Thompson et al., 1994). The result showed that all invertebrate VgRs including AaVgR, AgVgR, DmYPR, PaVgR, SiVgR and CeVgR formed a separate clade from vertebrate VgR and VLDLR (Fig. 3-5). The mosquito VLDLR (AaVLDLR) and the locust VLDLR (LmVLDLR) share 63% amino acid identity (Dantuma et al., 1999; Cheon et al., 2001) and clustered the vertebrate VgRs and VLDLRs into a larger clade, but were distantly related to insect VgRs (Fig. 3-5). Four insect VgRs of AaVgR, AgVgR, DmYPR and PaVgR have LL or LI amino acid sequence in the cytoplasmic tail as an internalization signal (first four VgRs in Fig. 3-5). However, all the other receptor members in the Fig. 3-5 have the typical LDLR internalization signal, NPXY. It will be interesting to see what type of internalization signal the honey bee VgR contains since the honey bee and the fire ant are both eusocial insects.

The ancient origin of LRPs was revealed by the discovery of a nematode LRP (Yochem and Greenwald, 1993). As a corollary, the discovery suggests that the original LDLR superfamily progenitor arose in an acoelomate. This ancestral molecule may have been a large LRP-like receptor from which smaller LDLR superfamily members

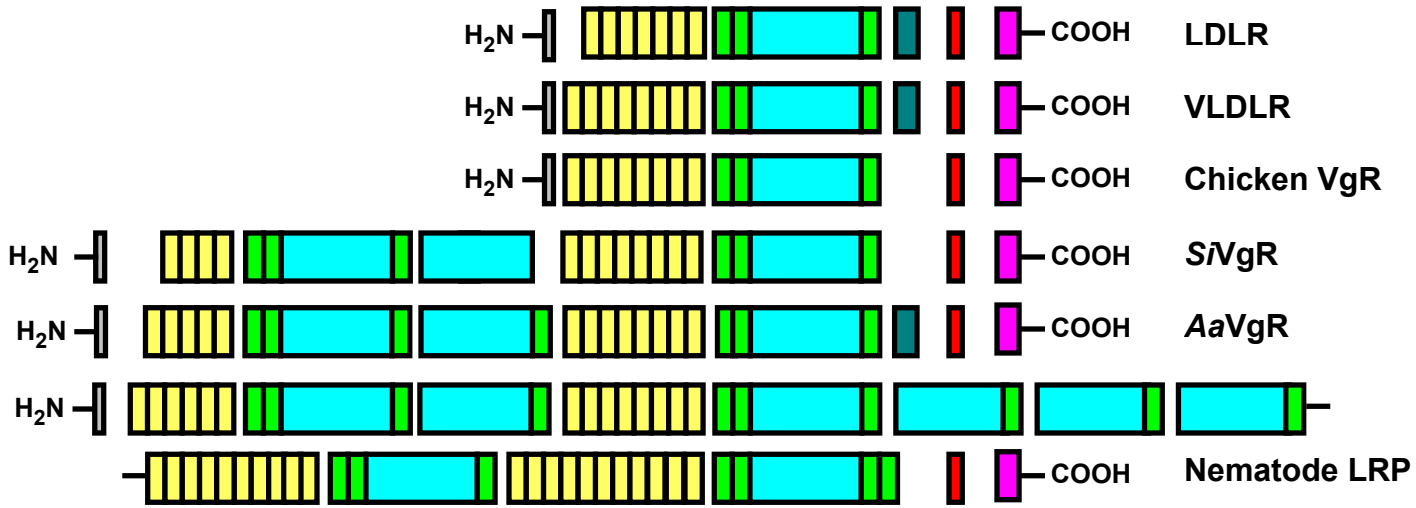


Fig 3-4. Schematic modular domain alignment among LDLR family members. Black boxes denote identical amino acids. Gray boxes indicate the signal peptide sequence. Yellow boxes indicate the ligand binding domains. Green boxes indicate the class B cysteine-rich repeats. Blue boxes indicate the YWXD repeats. The teal box indicates the O-linked sugar domain in *Aa*VgR only. Red boxes indicate the transmembrane region. Pink boxes indicate the cytoplasmic tail. (for citations, see text).

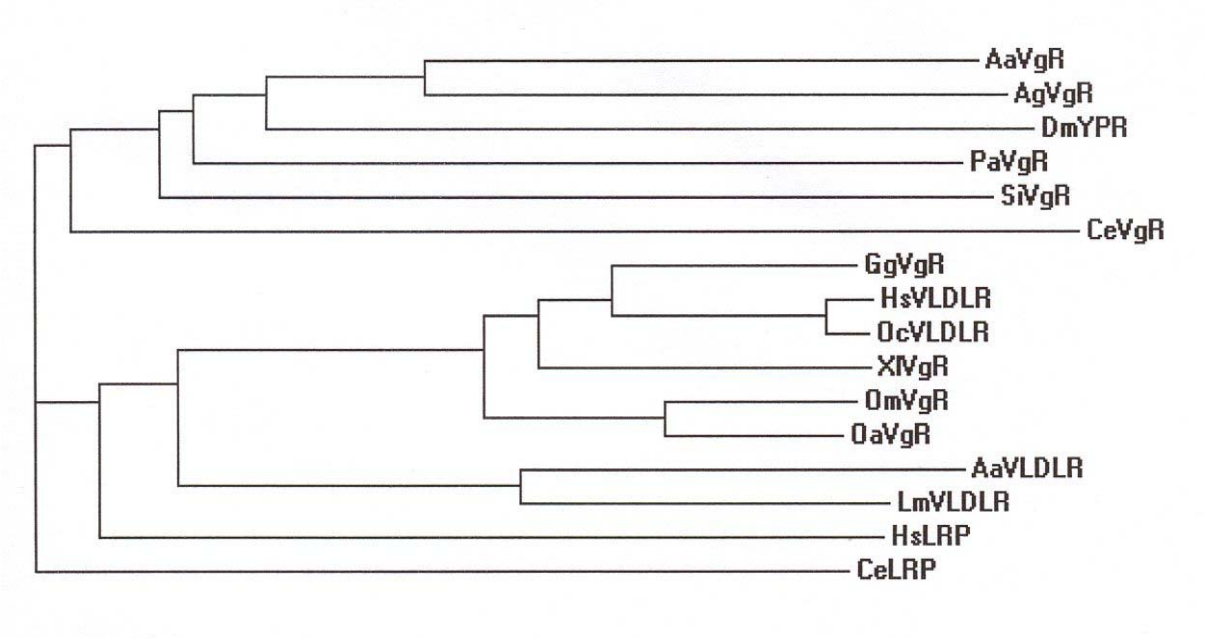


Fig. 3-5. The phylogram of sixteen LDLR superfamily members. The phylogram was generated based on amino acid seuquences alignment by the Clustal W computer program. *Aa*VgR= mosquito (*Aedes aegypti*) VgR (vitellogenin receptor); *Ag*VgR= mosquito (*Anopheles gambiae*) VgR; *Dm*YPR= fruit fly (*Drosophila melanogaster*) VgR; *Pa*VgR= roach (*Periplaneta americana*) VgR; *Si*VgR= fire ant (*Solenopsis invicta*) VgR; *Ce*VgR= nematode (*Caenorhabditis elegans*) VgR; *Gg*VgR= chicken (*Gallus gallus*) VgR; *Hs*VLDLR= human (*Homo sapiens*) VLDLR (very low-density lipoprotein receptor); *Oc*VLDLR= rabbit (*Oryctolagus cuniculus*) VLDLR; *Xl*VgR= frog (*Xenopus laevis*) VgR; *Om*VgR= rainbow trout (*Oncorhynchus mykiss*) VgR; *Oa*VgR= fish (*Oreochromis aureus*) VgR; *Aa*VLDLR= mosquito VLDLR; *Lm*VLDLR= locust (*Locusta migratoria*)VLDLR; *Hs*LRP= human LRP (LDLR Related Protein); *Ce*LRP= nematode LRP

are derived (Schonbaum, et al., 1995). Alternatively, it is hypothesized that vertebrate VgRs and VLDLR may represent primordial, multifunctional, yolk-protein precursor receptors from which more specialized receptors, such as LDLRs and LRPs, are derived (Hiesberger et al., 1995). Sappington *et al*. (1996) point to a third possibility that must be considered now, that the intermediate-sized insect VgRs represent the primordial type from which both the larger and smaller receptors are derived.

CHAPTER IV

LOCALIZATION OF THE FIRE ANT VITELLOGENIN RECEPTOR TRANSCRIPT

1. INTRODUCTION

Accumulation of massive amounts of the extra-ovarian yolk protein precursor, vitellogenin (Vg), by developing oocytes during oogenesis is a characteristic phenomenon of all oviparous animals (Stifani et al., 1990; Raikhel and Dhadialla, 1992). Internalization of Vg by the oocyte is achieved through receptor-mediated endocytosis. Consequently, the properties of vitellogenin receptors (VgRs) are of great interest to developmental biologists and much effort has been put into their study in several species.

The presence of a specific VgR in the oocyte membrane has been demonstrated in a number of insect species through studies of Vg uptake by cultured ovaries. Biochemical characterization of a few VgRs has been accomplished through their progressive isolation from solubilized membrane extracts. In all the insect VgRs, binding of Vg was saturable; ovary specific; showed high Vg affinity and was sensitive to changes in pH and Ca^{2+} concentration (Ferenz and Lubzens, 1981; Rohrkasten and Ferenz, 1985; Oliveira et al., 1986; Osir and Law, 1986; Kulakosky and Telfer, 1987; Kindle et al., 1988; Konig et al., 1988; Koller et al., 1989). Finally, VgR cDNAs were cloned from the fruit fly, *Drosophila menlogaster*, the yellow-fever mosquito, *Aedes aegypti*

(Schonbaum et al., 1995; Sappington et al., 1996) and the American cockroach, *Periplaneta americana* (accession number BAC02725).

To date, all cloned vertebrate and insect VgRs have shown that the VgR transcripts are expressed abundantly in ovaries. In this chapter, caste- and tissue-specific transcription of *Si*VgR are determined.

2. MATERIALS AND METHODS

2.1. Insects

S. invicta were reared as described in Chapter III.

2.2. Northern Blot Analysis

Total RNA was extracted for analysis of receptor transcript levels based on tissue, gender and caste specificities. To test the gender and caste specificity of the VgR gene expression, total RNA (10 μg) was extracted from whole workers, the fat body of males and from the ovaries and fat body of mated queens and alate females. Tissue specificity of VgR gene expression was determined from total RNA (10 μg) extracted from the head, flight muscle, midgut, fat body and ovary of alate virgin females. The RNA samples were electrophoresed in a 1.2 % agarose gel containing 0.7 M formaldehyde and 1X MOPS at 80 V for 90 min, then transferred to a positively charged nylon membrane (Hybond XL, Amersham Pharmacia, Piscataway, NJ) with 10X SSC as transfer buffer. The ~500 bp specific probe was prepared by digesting the 2.3 kb fragment with *Acs*I and *Hind*II restriction enzymes and labeled with ^{32}P. For labeling,

the Ambion DECAprime II Random Priming DNA labeling kit (Ambion, Austin, TX) was used with α-^{32}P dCTP (specific activity 3000 Ci/mmol, Perkin Elmer, Vellesley, MA) following the instructions manual. The membrane was hybridized with the ^{32}P-labeled probe at concentrations of $1x10^6$ cpm/ml of hybridization solution (Ultrahyb, Ambion, Austin, TX). Hybridization was performed at 42 °C for 16 h. Following hybridization, the membrane was washed twice for 5 min at low stringency (2X SSC, 0.1 % SDS) at 42 °C and twice for 15 min at high stringency (0.1X SSC, 0.1 % SDS) at 65 °C. After washing to remove non-specific radioactivity, the membrane was exposed to X-ray film and developed.

2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to determine tissue-specific transcription of *Si*VgR. Total RNA (1 μg) was extracted from head, flight muscle, midgut, fat body and ovaries of alate virgin females and from male fat body and whole workers using the guanidine-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987; Puissant and Houdebine, 1990). The total RNA was provided as templates for the reverse transcription (RT) using oligo-dT_{16} primer with GeneAmp® RNA PCR core kit (Perkin Elmer, Wellesley, MA). PCR amplification of *Si*VgR cDNA was performed using primer set 2.3-13 (forward), 5'-CAAGGAAGAAAATTCATTAACTACTATCACG-3' and 2.3-7R (reverse), 5'-TAAGGTGTCAAATGACCAGG-3'. The sequence was obtained from the *Si*VgR cDNA sequence (Chapter III). The gene for β-actin from the tick, *Boophilus microplus*, was used as a control to determine the uniformity of the RT

reactions. PCR amplification of β-actin cDNA was performed using primer set Act-3F (forward), 5'-TCCTCGTCCCTGGAGAAGTCGTAC-3' and Act-4R (reverse), 5'-CCACCGATCCAGACCGAGTACTTC-3' (Holmes et al., 2000). Reactions contained 1μl of the synthesized cDNA, 0.2 μM of each primer, 200 μM of dNTPs, 1X reaction buffer (Perkin Elmer) and 0.2 μl Taq polymerase in a final volume of 20 μl. PCR parameters were 94 °C for 2 min; 40 cycles of 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 2 min; 72 °C for 10 min.

3. RESULTS AND DISCUSSION

Northern blot analysis was carried out to study the caste, gender and tissue specificity of *Si*VgR gene expression. The *Si*VgR transcript was detected as a single 7.4 kb band restricted to the ovarian tissue of reproductive females -- both queens and alate virgin females (Fig. 4-1). Both *Aa*VgR and *Dm*YPR transcripts are similar in size, 7.3 kb and 6.5 kb respectively, to the *Si*VgR transcript and both transcripts are present in only the ovaries of their species (Schonbaum et al., 1995; Sappington et al., 1996). No *Si*VgR message was present in males or workers (Fig. 4-1A) and no *Si*VgR transcript was detected in total RNA from head, flight muscle, midgut or fat body (Fig. 4-1B). Major expression in the ovaries is observed for all cloned vertebrate and insect VgRs (Sappington et al., 1996; Schonbaum et al., 1995; Bujo et al., 1994; Okabayashi et al., 1996; Davail et al., 1998).

In *S. invicta*, the VgR transcript was present at higher levels in virgin alate females than in reproductively active queens (Fig. 4-1A). This observation appears opposite of

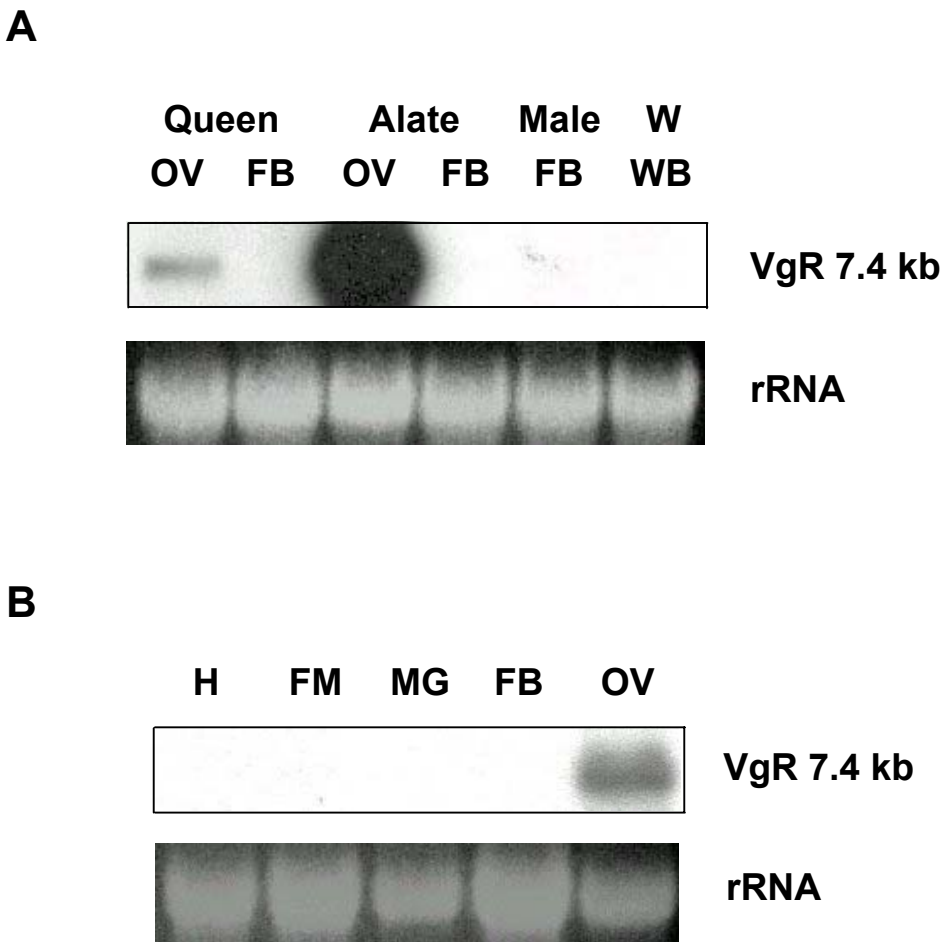


Fig. 4-1. Northern blot analysis of VgR in *S. invicta*. Total RNA (10 μg) was extracted from all castes and several major tissues. Blots were probed with a ^{32}P-labeled, *Si*VgR-cDNA fragment. Ribosomal RNA (rRNA) is shown as an internal control after staining with ethidium bromide. (A) Caste profile of *Si*VgR expression. (B) Tissue-specific expression of *Si*VgR transcripts in alate females. OV = ovary; FB= fat body; H = head; FM = flight muscle; MG = midgut; W = worker; WB= whole body.

what might be predicted; assuming its expression would be highest during active egg formation. However, in the chicken, the levels of VgR transcripts are high in the ovaries of immature hens, before producing fully grown oocytes (0.3 cm of oocyte), and the VgR transcript levels drop during the final growth phase of the oocytes (2.0 cm of oocyte) (Bujo et al., 1995a). In rainbow trout, VgR transcript levels are high in the stage that precedes the beginning of internalization of Vg into the oocytes, and there is no detectable VgR signal in the larger, fully vitellogenic ooctyes of rainbow trout (Davail et al., 1998). In mosquito, VgR mRNA is expressed at higher levels in both previtellogenic and early vitellogenic ovaries (Cheon et al., 2001). These common findings suggest that during the previtellogenic period, VgR transcripts are transcribed at a high level. During rapid yolk formation, vitellogenesis -- receptor-mediated uptake of Vg -- occurs at a maximal rate but without the synthesis of new VgR since VgRs recycle (Goldstein et al., 1985; Snigirevskaya et al., 1997).

Based on SDS-polyacrylamide gel electrophoresis analysis of hemolymph proteins, Vg is present in fire ant queens, alate virgin females and workers (Lewis et al., 2001). The presented northern blot analysis showed that there was *Si*VgR expression in queens and alate virgin females, but not in workers (Fig. 4-1A). The northern blot analysis was confirmed by RT-PCR, and the RT-PCR results showed that *Si*VgR was expressed only in ovaries of alate females, and not in the head, flight muscle, midgut or fat body. It was also not expressed in males and workers (Fig. 4-2). Therefore, the ovarian-specificity of *Si*VgR suggested that the function of *Si*VgR was related to reproductive processes, likely vitellogenesis. In the honeybee, *Apis mellifera*, workers contain a vitellogenin receptor in the membrane of the hypopharyngeal gland and Vg or Vg-derived products

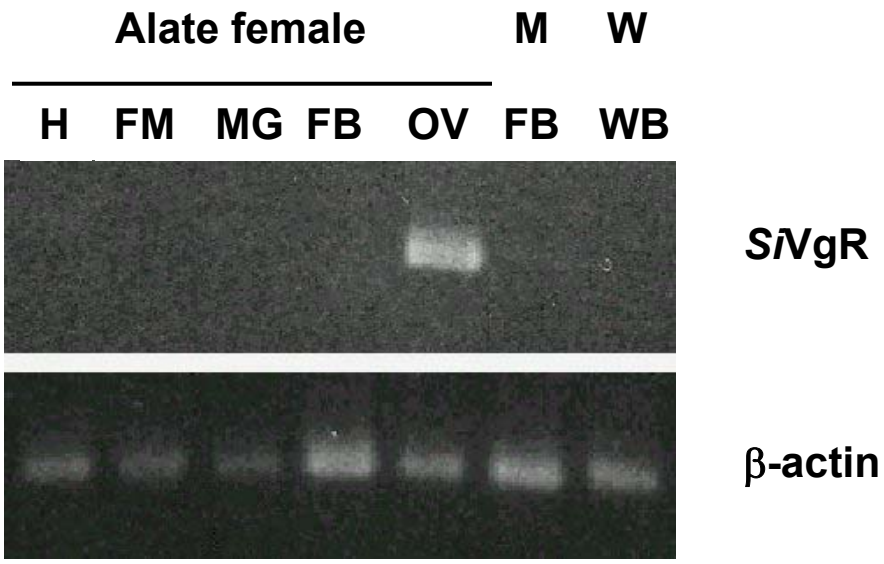


Fig. 4-2. RT-PCR analysis of VgR in *S. invicta* reveals receptor transcript amplification only from ovaries. cDNA was made from several major tissues and males and workers. β-actin is shown as an internal control after staining with ethidium bromide. H = head; FM = flight muscle; MG = midgut; FB = fat body; OV = ovary; M = male; W = worker; WB = whole body.

accumulate in the heads of young honeybee workers suggesting that Vg is used as food for the brood (Amdam et al., 2003). The function of Vg in *S. invicta* workers is not clear. It is possible that another VgR, different from the ovary-specific VgR of reproductives, may exist to internalize Vg in workers.

CHAPTER V

TRANSCRIPTIONAL REGULATION OF THE FIRE ANT VITELLOGENIN RECEPTOR

1. INTRODUCTION

Oogenesis is a complex process that involves several organs and is under hormonal control. One of the most common regulatory mechanisms for insect egg maturation is the stimulation of Vg synthesis by juvenile hormone (JH) (Engelmann, 1983; Koeppe et al., 1985). JH also plays a role in the regulation of Vg uptake by the oocytes (Bell and Barth, 1971; Davey, 1981).

In *Rhodnius prolixus*, JH stimulates both Vg synthesis by the fat body and Vg uptake by the ovaries. The main role of JH in Vg uptake is to bind to a putative JH receptor in the follicle cell membranes and stimulate the development of spaces between the follicle cells thus permitting Vg to access the oocyte -- a process known as patency (Davey, 1981; Ilenchuk and Davey, 1985). Patency results from a reduction in the volume of the follicle cells caused by JH activation of a membrane-bound Na^+-K^+ ATPase via a pathway involving protein kinase C (Davey, 1996).

In the locust, *L. migratoria*, both Vg synthesis and uptake are stimulated by JH. The follicle cells of *L. migratoria* respond to JH in the same manner as those of *R. prolixus* (Davey et al., 1993). The addition of the JH analog, methoprene, enhanced Vg uptake by more than 25% (Ferenz and Lubzens, 1981).

In *Aedes aegypti*, JH acts on the ovary at adult eclosion to make it competent to secrete ecdysteroids in response to the egg-development neurohormones released after feeding (Shapiro and Hagedorn, 1982). JH also acts on the fat body to make the fat body competent to convert the ovarian ecdysone into 20-hydroxyecdysone (20-OHE). The 20-hydroxyecdysone then stimulates the fat body to synthesize Vg (Flanagan and Hagedorn, 1977).

In *Drosophila melanogaster*, JH is also implicated in the regulation of vitellogenesis and vitellogenic oocyte developement (Jowett and Postlethwait, 1980; Wilson, 1982; Soller et al., 1999). JH stimulates yolk protein (YP) synthesis and uptake by the developing oocyte; ecdysteroids produced by follicle cells in the ovary and other tissues stimulate fat body YP synthesis (Koeppe et al., 1985; Bownes et al., 1993). This model is supported by observations of the JH-deficient mutant, *apterous*^{4}, in which little vitellogenesis occurs; a condition partially rescued by the application of the JH analog methoprene (Postlethwait and Weiser, 1973). Furthermore, when methoprene was applied to starved wild-type adults or isolated abdomens, conditions under which YP synthesis would be low, YP transcript became elevated (Jowett and Postlethwait, 1980). Methoprene also stimulated fat body YP transcript levels in fed flies (Bownes et al., 1987) further supporting the postulate that JH is required for vitellogenesis. Richard *et al*. (1998) presented evidence that this may not be due to the direct effect of the JH analog upon YP synthesis by fat body, but rather due to JH-stimulation of ovarian ecdysteroid synthesis, the latter in part responsible for YP production and uptake. Richard *et al*. (2001) presented further evidence that a role for JH is to regulate early

YP synthesis and uptake from follicle cells, and that in the absence of JH, late YP synthesis by the fat body is possibly under the control of ecdysteroids.

In species of primitively eusocial wasps and bumble bees, JH acts as a gonadotropin in the regulation of Vg synthesis in the fat body and the incorporation of Vg into developing oocytes in the ovary. JH may also play this role in the advanced eusocial fire ants (Bohm, 1972; Röseler, 1977; Röseler et al., 1980; Robinson and Vargo, 1997). However, there is no evidence that JH acts as a traditional gonadotropin in the honey bee and it clearly does not stimulate vitellogenin synthesis. Rather it appears to serve as an integrative element in social behavior and colony function (Hartfelder and Engel, 1998; Robinson and Vargo, 1997).

In this chapter, the temporal regulation of *Si*VgR transcription is determined in alate virgin females and mated queens. The regulatory roles of both juvenile hormone and 20-hydroxyecdysone in *Si*VgR transcription are examined in alate virgin females *in vitro*.

2. MATERIALS AND METHODS

2.1. Insects

S. invicta were reared as described in Chapter III.

2.2. Semiquantitative reverse transcription polymerase chain reaction (semi-QRT-PCR) for measuring the relative *Si*VgR transcriptional expression

Methods used for semi-QRT-PCR were similar to those described by Dozois *et al*. (1997). Total RNA (1 μg) was extracted from ovaries of different ages of alate virgin females and mated queens, and cultured ovaries from endocrine regulation studies using the guanidine-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987; Puissant and Houdebine, 1990). The total RNA was provided as templates for the reverse transcription (RT) using oligo-dT_{16} primer with GeneAmp® RNA PCR core kit (Perkin Elmer, Vellesley, MA). The final volume of RT reaction was increased to 50 μl with diethylpyrocarbonate (DEPC)-treated water.

PCR amplification of filamin cDNA was performed using primer set Fil-F, 5'-TTCAATGATCAGCACATCCC-3' and Fil-R, 5'-TTGTAACCCTCCTCGACCTC-3'. The sequence was obtained from *S. invicta* (Pietrantonio et al., 2002; Pietrantonio, unpublished). The filamin gene was used as a control to determine the uniformity of the RT reactions.

PCR amplification of receptor cDNA was performed using primer set 2.3-2 (forward), 5'-AAACGATGGCACGTACTG C-3' and 2.3-14 (reverse), 5'-AAGAAAGGCGTTGATAATTGCAGATGGC-3' (corresponding to amino acid sequence of 391-397 and 648-657, Chapter III, Fig. 3-3). Reactions contained one-tenth (5 μl) of the synthesized cDNA, 0.2 μM of each primer, 200 μM of dNTPs, 1X reaction buffer (Perkin Elmer) and 0.2 μl Taq polymerase in a final volume of 20 μl.

PCR parameters were 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 2 min; 72 °C for 10 min. Five microlitres of each reaction was electrophoresed on 1% agarose gel and stained with ethidium bromide. Gels were

photographed with Polaroid film and images were scanned with a Hewlett Packard ScanJet 5370C. The intensity of the bands was determined using Kodak 1D Image Analysis Software v3.5 (Kodak Scientific Imaging Systems, New Haven, CT). To compare the relative mRNA expression levels from each of the samples, the values were presented as the ratio of the band intensities of the receptor RT-PCR product over the corresponding filamin RT-PCR product.

2.3. Southern blot analysis

To assure that the RT-PCR products corresponded to the cloned receptor, each was re-amplified using the same primer set, 2.3-2 and 2.3-14, designed based on the sequence of the first cloned receptor PCR product obtained of 2.3 kb (Chapter III). PCR reactions were performed using the same reagents, concentrations and PCR parameters as the original RT-PCR. 5 µl of each reaction was run on a 1% agarose gel and stained with ethidium bromide. DNA was transferred to a positively charged nylon membrane (Hybond XL, Amersham, Pharmacia, Piscataway, NJ) with 10X SSC as transfer buffer following alkaline denaturation. After transfer, the membrane was illuminated with UV (300 nm) to fix the DNA. The membrane was hybridized with the ^{32}P-labeled *Si*VgR 2.3 kb cDNA fragment (Chapter III) corresponding to the region of RT-PCR products at a concentration of 1 x 10^6 cpm/ml of hybridization solution (Ultrahyb, Ambion, Austin, TX). Hybridization was performed at 42 °C for 16 h. Following hybridization, the membrane was washed 2 x 5 min at low stringency (2X SSC, 0.1% SDS) at 42 °C and 2 x 15 min at high stringency (0.1X SSC, 0.1% SDS) at 65 °C. After the membrane was

washed to remove non-specific radioactivity, it was exposed to X-ray film and developed.

2.4. Effects of age on *Si*VgR transcription

2.4.1. Alate virgin females

Reproductive pupae were maintained separately from the nest in a 24-well Falcon® tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ). Holes were melted into the lid of the plate with a hot needle to allow workers access to the pupae. The plate was placed within the colony so that the pupae could receive care from workers and be exposed to primer pheromone from queens, until eclosion. In order to determine age, following eclosion, alate virgin females were marked by spraying them with different colors of Krylon® enamel paint. The result showed that the colors of maize, dust pink, yellow, sun yellow, purple, grape and candy red were lasting long on alate virgin females. The marked alate females were returned to the colony.

The relative *Si*VgR transcriptional expression was determined in ovaries pooled from five insects. Ovaries were collected from untanned pupae (the pupal cuticle is white), pharate adults (the adult cuticle is completely developed and remains under the cover of the pupal cuticle), newly eclosed adults (day 0), and alate adults at 5, 10, 15, 20, 25, 30, 40, 50 and 60 days post-eclosion. Three replicate pools of five pairs of ovaries (3 replicates x 5 ovary pair each) were analyzed per age group using semi-QRT-PCR. The expression ratio (receptor/filamin) data were converted by the logarithm transformation (Montgomery, 1997) and analyzed by ANOVA followed by a Tukey

multiple comparison test to identify differences between individual means. Statistical analyses were performed using Prism^{TM} 2.0 (GraphPad Software, San Diego, CA).

2.4.2. Inseminated queens

Newly inseminated queens were field collected directly after a mating flight (College Station, TX). Queens were held separately in test tubes. The queens were held in a humid incubation chamber created by filling the test tubes half-full with water then plugging them in the middle with cotton and with another loose cotton plug at the top (Fig. 5-1). Ovaries were collected and pooled from ten queens each age point starting on the day of dealation (day 0) and on days 2, 4, 10, 15, 20, 25, 30 and 35 after mating; and on days 5 and 10 following eclosion of adult nanitics. Nanitics are a unique, uniformly small worker caste produced only in newly founded colonies (Vinson, 1997). Three replicate pools of ten pairs of ovaries (3 replicates x 10 ovary pair each) were analyzed per age group using semi-QRT-PCR. The expression ratio (receptor/filamin) data were converted by the logarithm transformation (Montgomery, 1997) and analyzed by ANOVA followed by a Tukey multiple comparison test to identify differences between individual means. Statistical analyses were performed using Prism^{TM} 2.0 (GraphPad Software, San Diego, CA).

2.5. Endocrine regulation study

Ovaries were dissected from newly-eclosed alate females (day 0) and incubated in a medium containing 20 amino acids at their physiological concentrations for *S. invicta* plus 19 mM glucose and 34 mM trehalose (Cônsoli and Vinson, 2002). Salts were

Fig. 5-1. The incubation chambers for newly mated queens. The chambers were created by filling the test tubes half-full with water then plugged newly mated queens in the middle with cotton and with another loose cotton plug at the top.

added equivalent to a cockroach Ringer (Keeley et al., 1995) and adjusted to pH 7.0. The medium was filter- sterilized and stored at 4 °C. Gentamicin (50 mg/ L) was added at the time of use.

Pools of seven pairs of ovaries were incubated in a disposable, sterile, polystyrene, 24-well Falcon® tissue culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) for 1 h at 27 °C. After 1 h pre-incubation, methoprene was added to the treatment well for a final concentration of 10^{-6} M using 1 μl of dimethylsulfoxide (DMSO) as a carrier. For controls, ovaries were incubated in plain culture medium or medium containing 1 μl of DMSO. All samples were incubated for 24 h at 27 °C. Six replicates of each treatment were performed, and the transcriptional expression of *Si*VgR was measured using semi-QRT-PCR. Mean values for experimental and control groups were based on six replicate samples (6 replicates x 7 ovary pair each). The expression ratio (receptor/filamin) data were converted by the logarithm transformation (Montgomery, 1997) and analyzed by ANOVA followed by a Tukey multiple comparison test to identify differences between individual means. Statistical analyses were performed using Prism™ 2.0 (GraphPad Software, San Diego, CA).

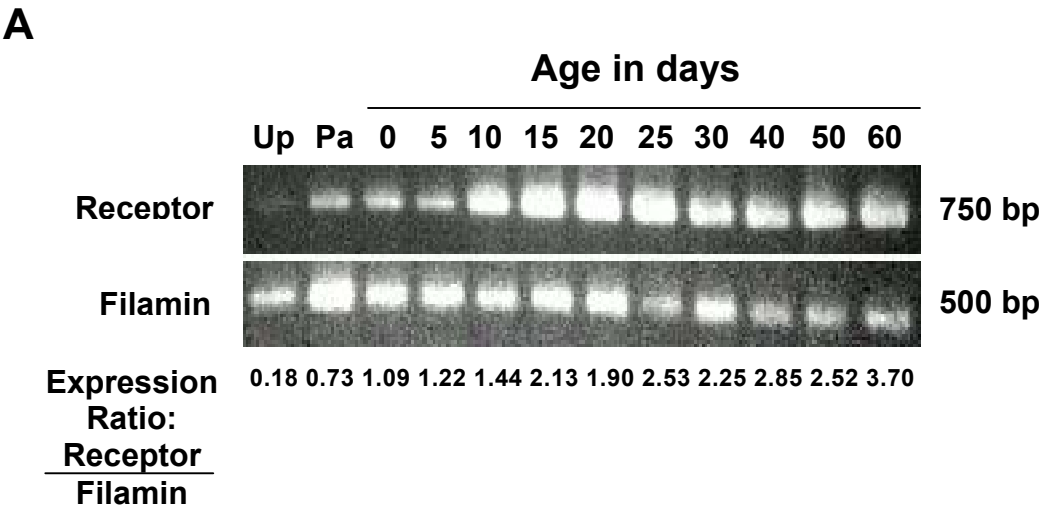
Experiments to assess the effect of ecdysone were performed in similar fashion with pools of seven ovary pairs as the experimental unit. 20-Hydroxyecdysone (20-OHE) (Sigma, St. Louis, MO) was applied to the same incubation system using ethanol as the hormone carrier. Three replicates of each treatment were performed. The expression ratio (receptor/filamin) data were converted by the logarithm transformation (Montgomery, 1997) and analyzed by ANOVA followed by a Tukey multiple

comparison test to identify differences between individual means. Statistical analyses were performed using Prism^{TM} 2.0 (GraphPad Software, San Diego, CA).

3. RESULTS AND DISCUSSION

Temporal expression of *Si*VgR mRNA was examined by semiquantitative RT-PCR. *Si*VgR cDNA samples were obtained from alate virgin females of various ages ranging from untanned pupae to 60 days after eclosion. The *Si*VgR sequence used for semi-QRT-PCR experiments and synthesis of the probe for Southern blot corresponds to the least similar region among VgRs and thus, it should identify *Si*VgR specifically (Chapter III, Fig. 3-3, arrows). The amount of amplified *Si*VgR cDNA (750 bp) produced by RT-PCR was compared with the amplification of *S. invicta* filamin (500 bp) (Fig. 5-2A). Filamin is an actin-binding protein in *Drosophila melanogaster* (Li et al., 1999b). During oogenesis filamin is in the ring canal structures that fortify arrested cleavage furrows and establish cytoplasmic bridges between cells of the germline (Li et al., 1999b). The expected, amplified 750-bp products were confirmed by Southern blot to hybridize to the cloned receptor (Fig. 5-2D). Fig. 5-2A and 5-2B shows that *Si*VgR transcripts were present in alate virgin females of all ages and continuously increased with age. *Si*VgR transcripts statistical significantly increased in old alate virgin females during the first 60 days of adult life (Fig. 5-2C, D 0 and D 60).

Semi-QRT-PCR was also performed to determine relative *Si*VgR transcription for different ages of field-collected, newly mated queens. Ovarian filamin again served as a control (Fig. 5-3A). Qualitative identification of amplified receptor transcripts (750-bp)



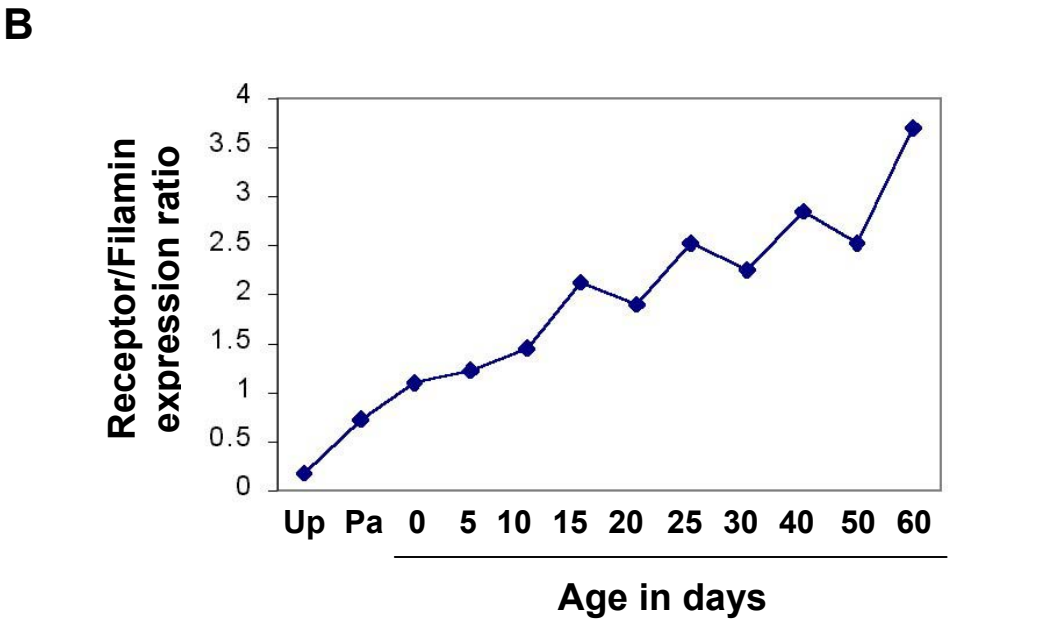
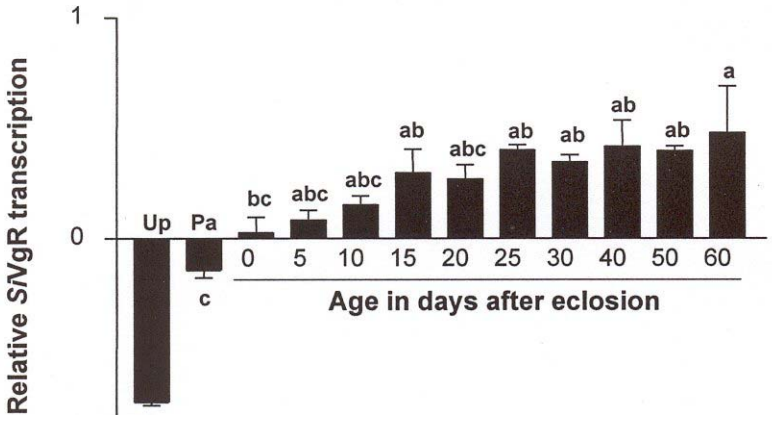


Fig. 5-2. Temporal profiles of VgR transcription in alate females of *S. invicta*. Alate females from a polygene colony were monitered following pupal eclosion. Up: untanned pupae, Pa: pharate-adult, and alate females (days 0-60) after eclosion. (A) Semiquantitative RT-PCR shows the relative amount of amplified receptor transcripts compared to amplified filamin transcripts for different ages. (B) Expression ratio (receptor/filamin) plotted against ages. (C) Statistical analysis results of relative *Si*VgR transcription with different ages. (D) Qualitative identification of amplified receptor transcripts. Panel 1: Agarose electrophoresis of PCR amplified receptor products for Southern blot. Panel 2: Autoradiograph of the Southern blot of DNA in panel 1. (-) = negative control containing DNA unrelated to the receptor.

C



D

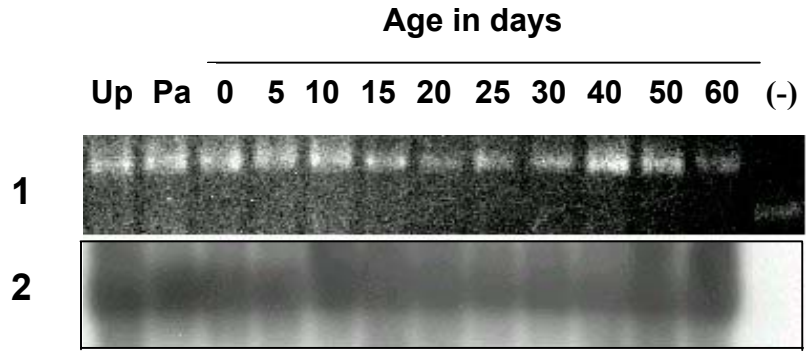
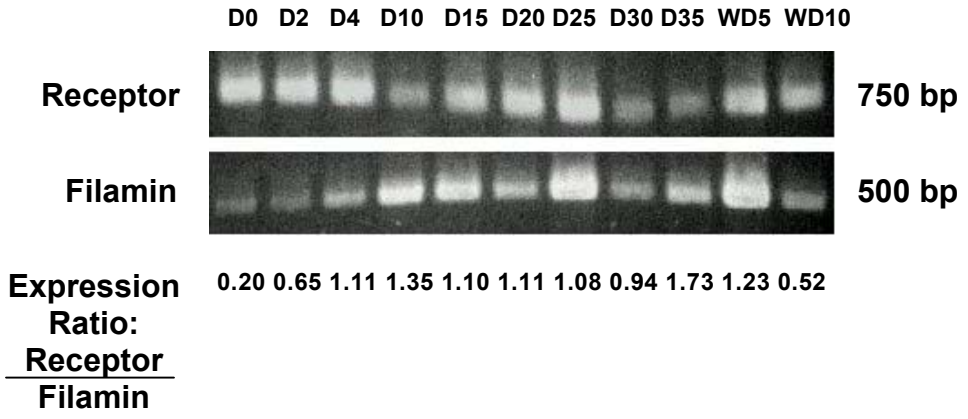


Fig. 5-2. Continued.

A



B

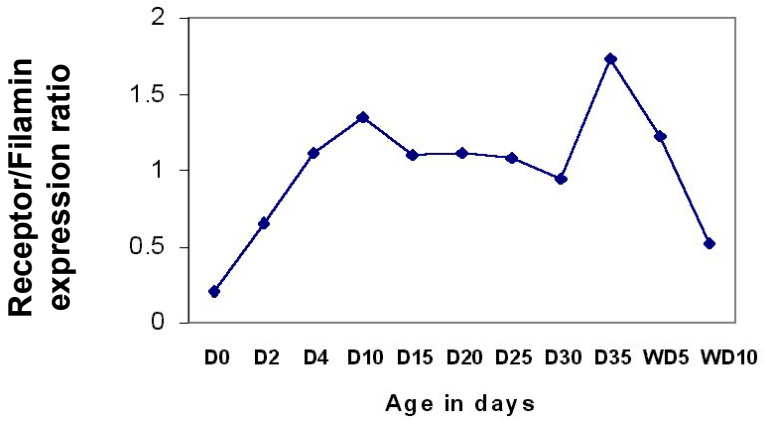
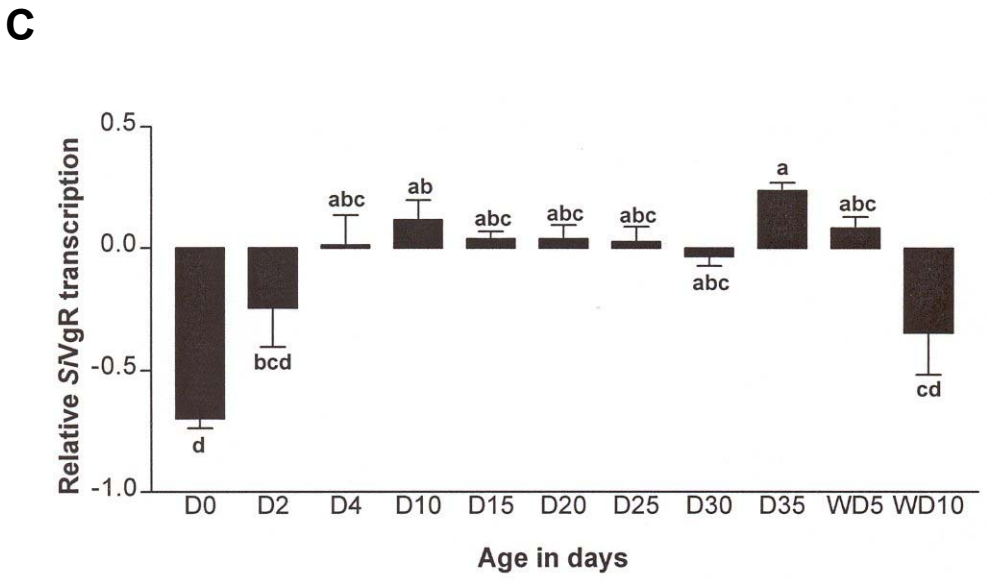


Fig. 5-3. Temporal profiles of VgR transcription in field-collected newly mated queens of *S. invicta*. Ovaries were collected at dealation (D0) and at intervals of 5 (WD5) to 10 days following nanitic emergence (WD10). (A) Semiquantitative RT-PCR shows the relative amount of amplified receptor transcripts compared to amplified filamin transcripts for different ages. (B) Transcriptional expression ratio (receptor/filamin) plotted against age. (C) Statistical analysis results of relative *Si*VgR transcription with different ages. (D) Qualitative identification of amplified receptor transcripts. Panel 1: Agarose electrophoresis of PCR amplified receptor products for Southern blot. Panel 2: Autoradiograph of the Southern blot of DNA in panel 1. (-) = negative control containing DNA not related to the receptor.



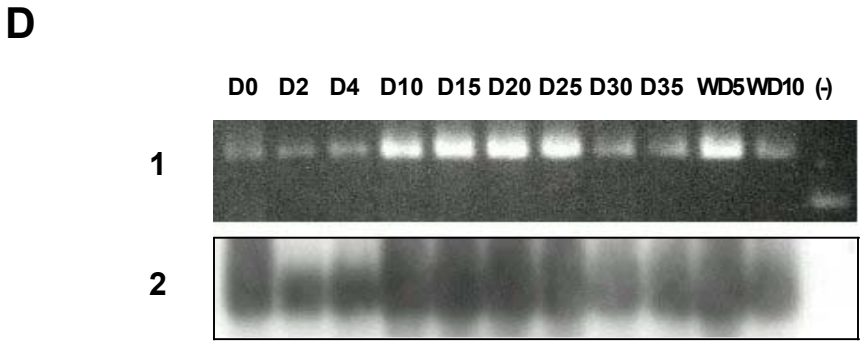


Fig. 5-3. Continued.

was confirmed by Southern blot analysis (Fig. 5-3D). The results showed that the *Si*VgR transcripts increased from days 0 to 10 and remained at the day-10 level until day 30. *Si*VgR transcripts increased by day 35 from day 10, then *Si*VgR transcripts declined following nanitic emergence (Fig. 5-3B). The reasons for this decline are unclear. The statistical analysis result showed that *Si*VgR transcripts in D 35 significantly increased from D 0, D 2 and 10 days after nanitic emergence (Fig. 5-3C). It is difficult to propose how *Si*VgR transcription is temporally regulated in newly mated queens, since the existing receptor proteins are believed to recycle. Comparison of relative *Si*VgR transcription between alate virgin females and newly mated queens showed that the level of *Si*VgR transcription was higher in all ages of alate virgin females than the level on D0 of newly mated queens. Untanned pupae showed similar levels of expression as in day-0 newly mated queens (Fig. 5-2A, 5-3A).

There is no data to indicate that alate virgin females are of a particular age when they mate and dealate to become queens. Whenever the environmental conditions are optimum, mating flights occur (Vinson, 1997). After mating, females land, dealate and lay 10-20 eggs within the first 24 hours. The low levels of *Si*VgR transcripts at dealation suggest that the *Si*VgR protein is either synthesized from existing transcripts or has already been synthesized and is ready to start uptake of Vg for yolk formation.

Ovaries from newly eclosed alate females were incubated *in vitro* at 27 °C for 24 h in the presence or absence of 10^{-6} M methoprene (a JH analog). Cultured ovaries were analyzed by semi-QRT-PCR using filamin as an internal control (Fig. 5-4A). The expected, amplified 750-bp products were confirmed by Southern blot to hybridize to the cloned receptor (Fig. 5-3C). Treatment of ovaries with methoprene increased *Si*VgR

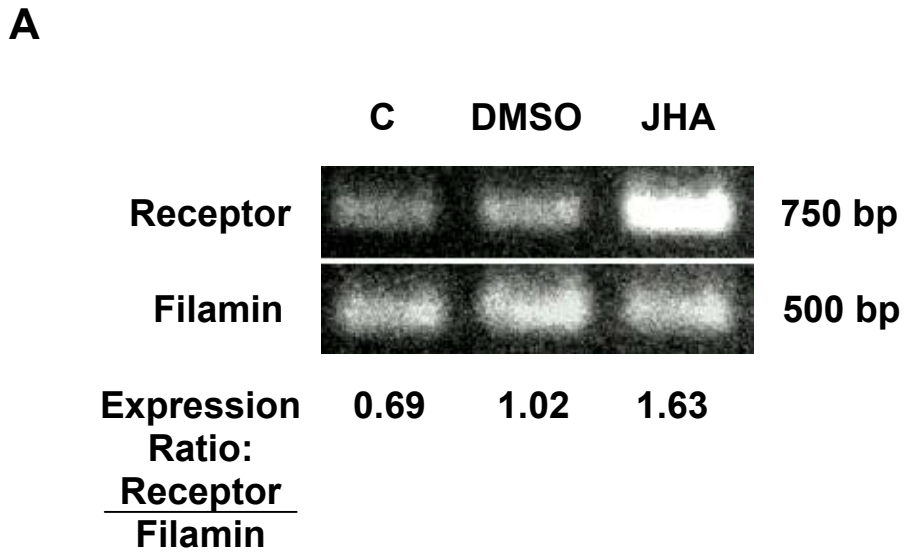


Fig. 5-4. Effect of the juvenile hormone analog, methoprene, on *Si*VgR transcription by *in vitro* ovaries from day-0 alate female *S. invicta*. C = control, DMSO = dimethyl sulfoxide, JHA = juvenile hormone analog, methoprene. (A) Semiquantitative RT-PCR shows the relative amounts of amplified receptor transcript compared to amplified filamin transcripts for several treatments. (B) The relative *Si*VgR transcription level of JHA-treated ovaries is significantly higher than control and DMSO-treated ovaries (Tukey multiple comparison test, $P < 0.05$). (C) Qualitative identification of amplified receptor transcripts. Panel 1: Agarose electrophoresis of PCR amplified receptor products for Southern blot. Panel 2: Autoradiograph of the Southern blot of DNA in panel 1. (-) = negative control containing DNA unrelated to the receptor.

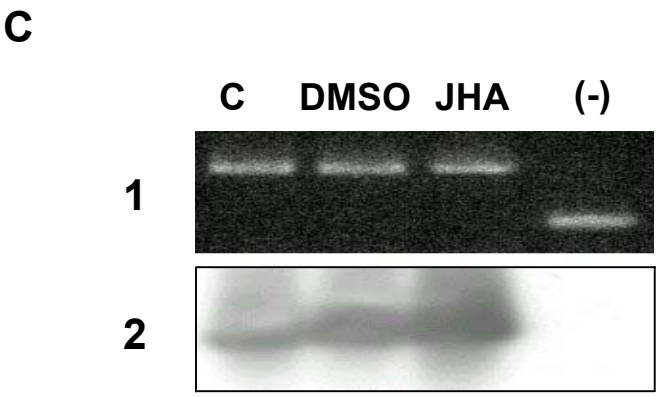


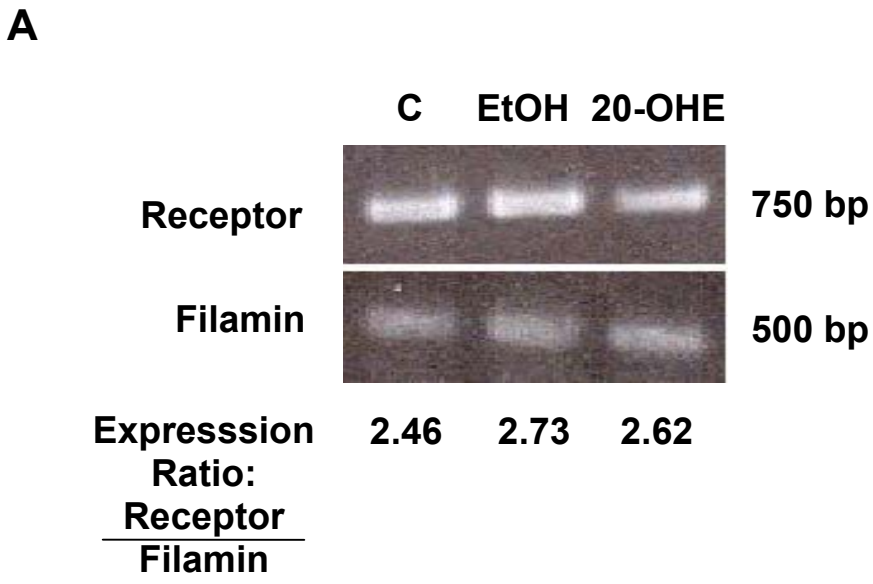
Fig. 5-4. Continued.

transcript levels by 1.4-fold compared with untreated control ovaries or 0.6-fold compared with DMSO-treated solvent control ovaries ($P<0.05$; Fig. 5-4B). The *in vitro* experiments suggested that JH may stimulate *Si*VgR gene expression.

We also investigated whether 20-OHE influenced *Si*VgR gene expression using the same *in vitro* system, but with ethanol as the hormone carrier. There was no significant difference between the 20-OHE-treated and control ovaries as measured by the log of the receptor/filamin transcription ratio (Fig. 5-5).

The regulatory role of 20-OHE in *Si*VgR transcription has still to be demonstrated by applying inhibitors of 20-OHE and examining the amount of *Si*VgR transcripts produced. For example several classes of compounds including cucurbitacins, withanolides, limonoids and stilbenoids antagonize the action of 20-OHE (reviewed in Dinan et al., 2001).

Several *in vivo* studies indicate that dealation and ovary development in fire ants are controlled by JH (Kearney et al., 1977; Barker 1978, 1979; Fletcher and Blum, 1983; Vargo, 1992; Vargo and Laurel, 1994; Burns et al., 2002), but *in vitro* experiments have not been performed. It is speculated that the likely mechanism that normally prevents alate females from dealating and initiating reproductive maturation in fire ants involves a primer pheromone produced by the queens (Fletcher and Blum, 1981; Vargo and Laurel, 1994) that may depress virgin alates JH titers (Vargo and Laurel, 1994). Earlier work (Vargo and Laurel, 1994; Lewis et al., 2001, 2003) demonstrated that vitellogenin was present in the hemolymph of alate female *S. invicta*, but without concomitant oocyte maturation. Topical treatment with methoprene induced alate females to develop their ovaries, even in the presence of a functional (pheromone-producing) queen (Vargo



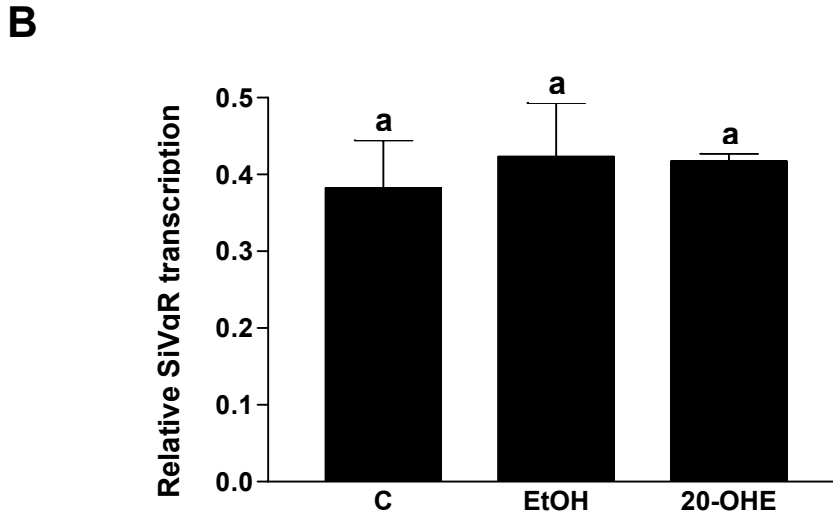


Fig. 5-5. Effect of the 20-hydroxyecdysone on *Si*VgR transcription by *in vitro* ovaries from day-0 alate female *S. invicta*. C = control, EtOH = ethanol, 20-OHE = 20-hydroxyecdysone. (A) Semiquantitative RT-PCR shows the relative amounts of amplified receptor transcript compared to amplified filamin transcripts for several treatments. (B) There are no significant differences among the relative *Si*VgR transcription levels for the various ovaries (Tukey multiple comparison test, $P > 0.05$).

and Laurel, 1994). The results of the present study together with our early work (Lewis et al., 2001; 2003) suggest that by depressing JH titers the queen pheromone prevents ovary development by blocking Vg uptake rather than preventing Vg synthesis.

Fletcher and Blum (1983) and Vargo (1992) hypothesized that fire ant queen primer pheromones prevent ovary development in alate virgin females and reproductively active queens by inhibiting JH synthesis. Further, Vargo and Laurel (1994) proposed a model for the mode of action of the fire ant queen pheromone that inhibits dealation and ovary development. In the model, the pheromone triggers antennal receptors which send inhibitory signals to the median neurosecretory cells in the brain. The suppressed median neurosecretory cells only weakly stimulate the corpora allata to synthesize JH and maintain low titers of the hormone. At low levels, JH stimulates vitellogenin synthesis in the fat body. In the absence of the pheromone, the fully active-neurosecretory cells send a stronger chemical (and/or neural) signal that triggers the corpora allata to produce larger quantities of JH. At higher titers, JH stimulates dealation and vitellogenin uptake by the ovaries (Vargo and Laurel, 1994).

In Dr. Keeley's laboratory earlier studies on Vg gene, methoprene treatment did not stimulate Vg transcription in both alate virgin females and mated queens (unpublished data). In the present studies, the JH analog stimulated the *Si*VgR transcription, even in alate females when *Si*VgR transcript was abundant (Fig. 5-4B). These two experimental results are compatible with the proposed model from Vargo and Laurel.

In order to prove the regulatory role of JH in *Si*VgR transcription, anti-JH agents could be applied to probe the relationship between JH and the *Si*VgR transcription and may suggest ways to suppress Vg uptake for *S. invicta* control. Precocenes,

fluoromevalonate, allylic alcohol derivates are all anti-JH agents. Precocene is a compound that destroys the source of JH, the corpora allata. Burns *et al*. (2002) demonstrated that the size of corpora allata was reduced in alate females of *S. invicta* from monogyne colonies after treatments with precocene II. Therefore, precocene might be applied to functional queens to lower their JH titers below the threshold needed for vitellogenesis. Precocene might also be applied to alate females to decrease the JH titer and prevent alate females from dealating and synthesizing Vg.

CHAPTER VI

CONCLUSION

Since its introduction in 1918, the red imported fire ant, *Solenopsis invicta*, has spread rapidly across the southeastern USA and has become a serious pest. Its high reproductive rate has equipped the fire ant with the ability to become a successful invader (Vinson, 1997). *S. invicta* forms monogyne and polygyne colonies with a single queen or with multiple functional (egg-laying) queens, respectively. Although individually the queens of polygyne colonies consistently lay fewer eggs than the queens of monogyne colonies, they collectively produce more eggs per colony (Fletcher, et al., 1980; Vargo and Ross, 1989). Queens of one polygyne colony can produce an average of 355 eggs within five hours (Fletcher, et al., 1980). This high reproductive capacity is the one of main reasons why fire ants are so difficult to control; thus, inhibiting egg maturation is a promising strategy for fire ant control.

In insects, during egg maturation, the major yolk protein precursor, vitellogenin (Vg), is synthesized by the fat body and secreted into the hemolymph. The internalization of Vg into the growing oocytes is achieved through receptor-mediated endocytosis. In insects, various hormones and environmental cues regulate egg formation. However, species differ greatly in which hormones they use for regulation of egg formation. In many species, the synthesis or uptake of Vg is stimulated by juvenile hormone (JH). A notable exception is the Diptera, in which Vg synthesis is stimulated by ecdysteroids.

However, the presence of Vg does not necessarily equate with reproductive competency. In *Apis mellifera*, Vg is produced by worker bees during the nurse stage and occurs in haploid drones as a minor fraction (Trenczek and Engels, 1986; Trenczek et al., 1989). In *S. invicta*, Vg is present in the hemolymph of all female caste members, including workers, alate virgin females and mated queens (Lewis et al., 2001). Alate virgin females attain Vg titers comparable to functional queens, but without exhibiting ovarian maturation, suggesting that reproduction may be controlled by Vg uptake and not by Vg synthesis (Vargo and Laurel, 1994).

This dissertation reports the cloning of the first hymenopteran vitellogenin receptor (VgR) cDNA from the imported fire ant, *S. invicta*. The complete 5764 bp cDNA encodes a 1782-residue protein with a predicted molecular mass of 201.3 kDa (= *Si*VgR). Northern blot analysis demonstrated that the 7.4-kb *Si*VgR transcript was present only in ovaries of reproductive females (both alate virgin females and mated queens). The temporal profile of transcriptional expression showed that *Si*VgR mRNA increased with age in alate virgin females and could be up-regulated by methoprene, a juvenile hormone (JH) analog *in vitro*. This suggests that the *Si*VgR gene may be JH regulated.

Only the *Si*VgR cDNA sequence is reported here. It is important for future work to obtain the promoter region of the *Si*VgR gene to investigate the relationship between JH and possible transcription factors, and analyze potential regulatory elements. This provides clues to how JH affects *Si*VgR expression.

This research is the first to show induction of gene transcription *in vitro* by juvenile hormone in hymenopteran insects. This *in vitro* system is a powerful system to define

the regulatory role of JH in genes involved in oocyte maturation. It can avoid the influence of endogenous JH sources and potential inhibitors of JH. This system can be applied to test the regulation of other genes by JH.

Vargo and Laurel (1994) proposed that queen pheromone depresses JH production so that JH titers are below the threshold needed to stimulate Vg uptake, but above the level required to induce Vg synthesis. The present data show that even though the *Si*VgR transcript was abundant in alate virgin females, methoprene treatment still had the capacity to increase *Si*VgR transcripts (Fig. 4-1A and 5-3A). These results are compatible with the proposed hypothesis (Vargo and Laurel, 1994).

In most insects, the onset of vitellogenesis is characterized by the opening-up of the spaces between follicle cells, a phenomenon called patency, and JH stimulates the formation of spaces between follicle cells (Davey, 1981). In alate virgin female *S. invicta*, the JH titer is high enough to stimulate Vg synthesis, but not enough to stimulate Vg uptake. In addition to the regulation of *Si*VgR, is the JH titer above the threshold also needed to stimulate patency? Transmission electron microscopy can be performed to observe ovary structure to compare the size of spaces between the follicle cells of alate females and queens.

Vg receptors have to be incorporated into the plasma membrane of the oocyte to function properly. In *D. melanogaster*, the yolk protein receptor is expressed in oocytes during the previtellogenic period. These receptors are distributed throughout the cytoplasm of the oocyte. During vitellogenesis when Vg is being accumulated, the receptors are enriched and localized at the plasma membrane of the oocyte (Schonbaum et al., 2000). This same pattern of expression and localization of VgR is present in

chicken (Bujo et al., 1994). Therefore, the question is whether the fire ant Vg receptor is being expressed in alate virgin females. If so, where is the receptor localized within the non-vitellogenic oocytes?

In order to answer these questions, it is essential to produce a specific anti-*Si*VgR antibody. Expression and localization of *Si*VgR protein in mated queens and alate virgin females could be determined by western blot analysis and immunocytochemistry, respectively, using the anti-*Si*VgR antibody.

There are four repeats in the first ligand-binding domain of *Si*VgR and it is different from five in the first ligand-binding domain of the mosquito and fruit fly (Fig. 3-2). It would be interesting to test the affinity of the fire ant, mosquito and fruit fly VgR first ligand-binding domain for Vg binding, because they are unique among the know LDLR superfamily members. The NPXY sequence is necessary for coated pit-mediated internalization. It may be possible to mutate the NPXY sequence to disrupt the internalization process (Lehrman et al., 1985; Chen et al., 1990) to disrupt Vg accumulation in the fire ant oocyte.

Now that we know *Si*VgR is a member of the LDLR superfamily it will be of great interest to determine if *Si*VgR recognizes more than one ligand, like other LDLR members. Especially, studies from our laboratory (Lewis et al., 2003) and another laboratory (Dr. Craig Coates, Department of Entomology, Texas A&M University, College Station, TX) indicate that there are probably three Vg genes. Does the cloned *Si*VgR bind and internalize all three Vgs? Or there are three different Vg receptors for different Vgs? Does the oocyte express Vg by itself? These are all interesting topics for future research.

Based on the results of the present study and the primer pheromone hypothesis (Vargo and Laurel, 1994), a general hypothetical model for JH regulation of egg maturation of fire ants is illustrated in Fig. 6-1. Mated queens produce the primer pheromone to regulate the JH titers in alate virgin females. The JH titer is enough for Vg synthesis and *Si*VgR transcription in alate females. The possible linkage between the JH titer and Vg uptake could be: 1) the expression and secretory pathway of *Si*VgR to the plasma membrane of oocytes; 2) the machinery of the endocytotic pathway; 3) the regulation of patency.

The JH titer is supposedly higher in mated queens than in alate females. However, the northern blot analysis (Fig. 4-1) showed that level of *Si*VgR transcripts is lower in mated queen than in alate females. I would hypothesize that in mated queens the effect of JH regulation switches from transcription to the *Si*VgR protein biology (synthesis, post-translational modifications, membrane targeting, and so on) or the regulation of patency. JH may bind to the JH receptors on the plasma membrane of follicle cells to stimulate the patency in mated queens (Davey, 1981; Ilenchuk and Davey, 1985) and a question derived from this is whether the membrane JH receptor appears in alate females. JH may also regulate the expression and localization of *Si*VgR protein in the oocyte plasma membrane.

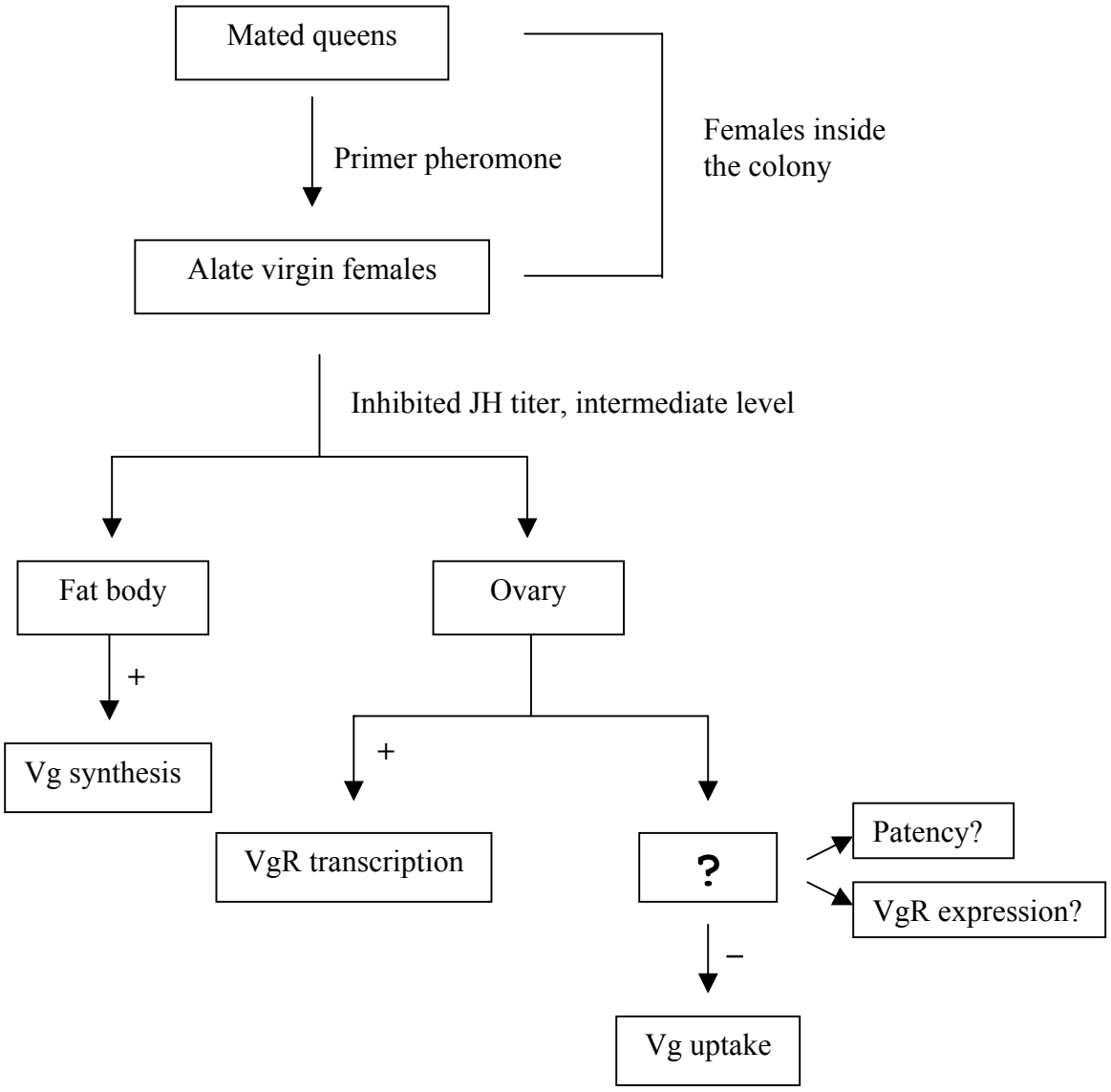


Fig. 6-1. A general hypothetical model of juvenile hormone (JH) regulation of the egg formation in alate virgin female *S. invicta*. Mated queens produced the primer pheromone to decrease the JH titer in alate females that are inside the same colony. The inhibited JH titer is enough for Vg synthesis by fat body and *Si*VgR transcription in the ovary. The possible answer of the question box, that is what determines the beginning of Vg uptake, could be the presence of patency or the expression and localization of *Si*VgR

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